ISOLATION, PURIFICATION AND CHARACTERIZATION OF ZN-BINDING FACTORS ASSOCIATED WITH THE CITRUS BLIGHT SYNDROME

By

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To Bill, for his love
and unwavering loyalty.

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KEY TO ABBREVIATIONS

A-254 nm	Absorbance at 254 nm
A-280 nm	Absorbance at 280 nm
ACTH	Adrenocorticotropic hormone
ATP	Adenosine 5'-triphosphate
Cys	Cysteine
CTP	Cytidine 5'-triphosphate
DHAP	Dihydroxyacetone phosphate
DNA	Deoxyribonucleic acid
DNP	2,4-dinitrophenol
DEAE	Diethylaminoethyl
DPP	Differential pulse polarography
F-1,6-P	Fructose-1,6-phosphate
F-6-P	Fructose-6-phosphate
G-1-P	Glucose-1-phosphate
G-6-P	Glucose-6-phosphate
Glu	Glutamic acid
Gly	Glycine
GTP	Guanosine 5'-triphosphate
IAA	Indole 3-acetic acid
IEC	Ion exchange chromatography
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dihydroxynucleotide
NDP	Nucleotide 5'-diphosphate

NMP	Nucleotide 5'-monophosphate
NTP	Nucleotide 5'-triphosphate
OAA	Oxaloacetic acid
PAGE	Polyacrylamide electrophoresis
PEP	Phosphoenolpyruvate
3PGAld	3-phosphoglyceraldehyde
PVPP	Polyvinylpolypyrrolidone
RCC	Residual complexation capacity
RuBP	Ribulose bisphosphate
SOD	Superoxide dismutase
TEMED	N,N,N',N'-tetramethylethylenediamine
t-RNA	transfer ribonucleic acid
TCA	Trichloroacetic acid
TCC	Total complexation capacity
Tris	Tris[hydroxymethyl]-aminomethane
UTP	Uridine 5'-triphosphate

Abstract of Dissertation Presented to the Graduate School
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Redistribution of Zn within citrus trees has been found associated with the predecline and decline stages of the disease, citrus blight. An accumulation of Zn occurs in trunk phloem above the bud union in blight-affected trees at the predecline and decline stages. This study determined that Zn-binding factors also were associated with the occurrence of citrus blight in predecline and decline stages, and that the Zn-binding factor accumulated in trunk phloem above the bud union. The Zn binding factors were found to be highly anionic and composed of at least 4 anionic species by DEAE Sephadex-A-50 ion exchange chromatography and polyacrylamide gel electrophoresis. Two anionic species were associated with the healthy tree, while all 4 were associated with the occurrence of citrus blight. The metal- binding factors displayed absorbance at 254 nm, indicating their metal-thiol chromophore character and they lacked absorbance at 280 nm. These are

common traits of certain metal-binding molecules, phytochelatins. They were capable of removing Zn from a Zn-containing solution, as assayed by differential pulse polarography (DPP). The fractions from chromatography which displayed absorbance at 254 nm and which were capable of removing Zn from a Zn-containing solution also contained elevated levels of Zn. These characteristics combined are circumstantial evidence that this metal binding factor is a Zn-binding factor and a phytochelatin. All the Zn-binding factors were isolated with an apparent molecular weight of 4kd, using Sephadex G-50, fine, gel filtration.

Phytochelatins are reported to be involved in heavy metal homeostasis in plants. In the case of citrus blight, the Zn-binding factors may be produced in excess, or new forms of these Zn-binding factors may be produced and remove Zn from availability for Zn-requiring enzymes. In this way Zn-binding factors may alter Zn metabolism of the tree somehow contributing to the occurrence of citrus blight, which is manifested as a decreased water conductivity and general decline of the tree.

CHAPTER 1
LITERATURE REVIEW

Tree Physiology of Citrus and Citrus Blight

Citrus blight is a major cause of tree loss and reduced fruit yield in citrus production areas in Florida (Smith, 1974) and around the world, with the exception of citrus production areas in Mediterranean climates (Wutscher et al., 1977; Graca and van Vuuren, 1979; Childs, 1979b; Lima, 1982). It is second only to freezes as a cause of mature tree loss in Florida. Several comprehensive reviews of blight literature are available (Childs, 1979a, 1979b; Cohen, 1968; Rhoads, 1936; Smith, 1974; Smith and Reitz, 1977).

The blight disorder has received attention by citrus growers and scientists in Florida for nearly 100 years. The earliest descriptions of citrus blight were reported in 1891 by Underwood and 1896 by Swingle and Webber (Rhoads, 1936). Two possible causes of blight were cited: 1) local soil conditions or 2) a contagion (Rhoads, 1936). The latter was considered less likely because no organism had been found associated with blight and it had not been transmitted in budwood to trees propagated from buds from wilted portions of typical blighted trees.

The cause of blight still remains unknown. The disease is regarded as an extremely complex decline problem due to the fact that on occasion it appears to be influenced by cultural, climatic and edaphic factors (Young et al., 1980; Nemec et al., 1982; Wutscher and Hardesty, 1979; Wutscher, 1981b). Additionally, there is evidence of transmission

by continuous root grafts (Tucker et al., 1984; Tucker et al., personal communication, 1986); however, no transmitted organism has been isolated. With a scattered array of influences and effects of blight, a single cause seems contraindicated. Yet the fact that it is transmissible suggests a single cause. Because symptom development is the best documented information available regarding citrus blight, a system of working backwards through symptom development ultimately to the cause has seemed the most suitable approach. There are three recent findings which appear to hold promise in understanding the citrus blight syndrome.

1) Water conductivity decreases with progression of the blight syndrome (Young and Garnsey, 1977). This decrease is due to increased plugging of the inner xylem vessels associated with wilt and the decline stage (Brlansky et al., 1984). Amorphous plugging is responsible for the reduced H_2O conductivity which leads to wilt.
2) Zinc accumulates first in the phloem and then in the outer wood in the very early stages of decline, prior to xylem plugging (Albrigo et al., 1986).
3) Blight is transmitted from blight-affected trees to healthy trees through root grafts in 2-3 years. In contrast, in side-by-side plantings of non-grafted healthy and blight-affected trees, the healthy trees do not develop blight in that period (Tucker et al., 1984). Transmission is indicated when trees show Zn accumulation followed by reduced H_2O conductivity.

Of these three recent findings, most significant (because it is the first known change) is the finding that Zn accumulates first in the phloem and then in the outer (youngest) wood of the trunk prior to the development of xylem plugging in the inner (older) trunk wood (Albrigo et al., 1986; Young et al., 1980). This finding suggests the Zn redistribution may result in Zn deficiencies observed in other portions of the tree during the development of the blight syndrome. Zinc deficiency patterns in leaves can accompany Zn accumulation in the phloem and wood if trees are not provided foliar applications of Zn (Rhoads, 1936).

There are other alterations in metabolite levels which occur either concurrently or following wilt of the tree. Wood pH (Wutscher et al., 1983) and wood phenolics (Wutscher et al., 1977; Wutscher, 1981a; Wutscher, 1983) increase as do scion bark and leaf proline (a typical water stress response) and trunk nitrogen (an indication of metabolite imbalance) (Hanks and Feldman, 1974; Syvertsen and Albrigo, 1984). ATP, carbonic anhydrase (Bausher, 1979) and IAA oxidase (Bausher, 1982) activities decresse. Carbonic anhydrase and IAA oxidase are Zn-requiring enzymes (Randall and Bouma, 1973; Lindskog, 1983; Vallee, 1983). Decreased activities of these enzymes may be a secondary response to Zn redistribution within the tree. The other metabolite changes also may be secondary responses due to stresses brought about by the Zn redistribution.

The reduction of feeder roots beneath the tree is an additional change occurring in blight-affected trees (Burnett et al., 1982; Nemec et al., 1982). This occurs during the later stages of the blight syndrome (Albrigo et al., 1986; Cohen, 1968). Such root loss may be due

to a secondary infection of Fusarium solani Mart. It has been suggested that reduced carbohydrate supply to the roots and the consequent reduction in root vigor would be permissive to such secondary infection (Graham et al., 1983).

Several mineral element concentrations are altered as well (Wutscher and Hardesty, 1978; Williams and Albrigo, 1984). All elemental changes with the exception of Zn occur either concurrently or after visible wilt (Albrigo et al., 1986). Zinc accumulates first in the active phloem and then in the outer wood. The Zn redistribution occurs one to three years prior to xylem plugging which develops in the inner wood and is responsible for H_2O deficits and wilt symptoms (Wutscher et al., 1982; Albrigo et al., 1986). Zinc accumulation occurs in a different location from the xylem plugging. Thus Zn accumulation and xylem plugging are not directly linked in symptom development, in terms of location and time. Zinc accumulation may also be a secondary physiological response to a blight inducing agent which may be transmissible through root grafts. However, it is the earliest known response associated with the development of blight since it occurs prior to reduced water conductivity (Tucker et al., 1984: Albrigo et al., 1986). Therefore the mechanism which mediates this redistribution needs to be studied.

Zinc Metabolism

Many enzymes in higher plants have been determined to require the presence of Zn as a cofactor for their optimum activity. Zinc is a IIB transition metal with 2s electrons which combine in the 2+ oxidation state. Zinc is neither oxidized nor reduced in biological reactions.

Zinc is considered to be poised for its intended catalytic function in enzymes due to its chemical characteristics and because it is capable of octahedral, tetrahedral and pentagonal coordination (Vallee, 1983). The first nutritional study of Zn was performed by Raulin in 1869 when he demonstrated the necessity of Zn for the growth of Aspergillus niger Mich. Keilin and Mann (1940) isolated the first Zn-requiring enzyme, carbonic anhydrase, from mammalian erythrocytes. They demonstrated that the protein contained 0.33% Zn. Since that time almost 200 Zn-enzymes have been isolated from plant and animal systems (Vallee, 1983). A list of some of the Zn-requiring enzymes and their roles in plant metabolism is given in Table 1.

Some of the key areas where Zn imbalance within the plant may have significant effects on plant metabolism are discussed below. The information given in the following paragraphs was taken from Goodwin and Mercer (1983), except where noted.

Superoxide dismutase (SOD) is important for quenching the free radicals that are naturally generated in the high energy light reactions of photosynthesis. If the superoxide anionic free radical is not reduced via the SOD mediated reaction ($O_2^{-\cdot} \xrightarrow{2H_2O} H_2O_2 + O_2$), the cell's membranes (especially chloroplast membranes) would be irreversibly damaged and photo-oxidation would occur. This refers to the destruction of the "photosynthetic machinery." The energy for photosynthetic carbon reduction, which fuels carbon biosynthesis, would not be available without the completion of the photosynthetic light reactions.

Carbonic anhydrase catalyzes the attainment of equilibrium between CO_2 (substrate for RuBP carboxylase) and HCO_3^- (substrate for PEP carboxylase) (Randall and Bouma, 1973; Goodwin and Mercer, 1983). In

Table 1.
Zinc metallo-enzymes involved in plant metabolism

Zinc Metallo-Enzyme	Enzyme-Role in Plant Metabolism	Zn-Role	References[b]
Alcohol Dehydrogenase (1.1.1.1)	Acetaldehyde $\xrightarrow{NADH\ NAD^+}$ Ethanol	A	1,2,3
Malate Dehydrogenase (1.1.1.37)	Malate ⟶ OAA [TCA Cycle]	?	2,3,4
Glyceraldehyde 3-Phosphate Dehydrogenase (1.2.1.12)	3PGAld ⟶ 1,3 Diphosphoglyceric Acid	?	2,3,4
Superoxide Dismutase (1.15.1.1)	$O_2^{-\cdot} \xrightarrow{H_2O} H_2O_2 + O_2$	?	2,3
Glutamate Dehydrogenase (1.4.1.3)	α-ketoglutarate + NH_4^+ ⟶ Glutamate	?	2,3,4
IAA Oxidases	IAA ⟶ Indolealdehyde	B	2,3
Aspartate Transcarbamylase (2.1.3.2)	Carbamoyl phosphate $\xrightarrow{Pi}$ Carbamoylaspartate	C	2,3
Phosphoglucomutase (2.7.5.1)	G-1-P ⟶ G-6-P	?	2,3
RNA Polymerase (2.7.7.6)	RNA synthesis	A	2,3,5
DNA Polymerase (2.7.7.7)	DNA replication	A	2,3,5
Fructose-1, 6-Bisphosphatase (3.1.3.11)	F-1,6-P ⟶ F-6-P [Gluconeogenesis]	B	2,3

Table 1 cont'd.

Zinc Metallo-Enzyme	Enzyme-Role in Plant Metabolism	Zn-Role	References[b]
α-Amylase (3.2.1.1)	Degradation of α-1-4 glucosidic linkages of starch	C	2,3
α-D-Mannosidase (3.2.1.24)	Hydrolysis of mannosides to mannose	?	2,6
Phospholipase C (3.1.4.3)	Degradation of acyl groups of polar lipids	A	2,3
Neutral Proteases	Degradation of neutral proteins	A	2,3
Nucleotide Pyrophosphatase (3.6.1.1)	NMP + NTP $\longrightarrow$ 2NDP (NTP precursor)	A	2,3
F 1,6-P Aldolase (4.1.2.13)	DHAP + 3-PGAld $\rightarrow$ F 1,6-P	A	2,3,4
Carbonic Anhydrase (4.2.1.1)	$CO_2 + H_2O \rightarrow H_2CO_3 \rightarrow H^+ + HCO_3^-$	A	1,2,3,7
S-Aminolaevulinic Acid Dehydratase (4.2.1.24)	Chlorophyll Biosynthesis	A	2,3
t-RNA synthetase	t-RNA synthesis	?	2
Tryptophan Synthase (4.1.2.20)	Indole-3-glycerol phosphate $\longrightarrow$ Tryptophan	A	5
Enolases (4.2.1.11)	2-Phosphoglyceric acid $\rightarrow$ PEP	?	5

[a]Indicates the role of Zn in the enzyme's activity: A- catalytic; B- regulatory; C- structural; ?- unknown.

[b]References given by number: 1- Bonner and Varner, 1973; 2- Vallee, 1983; 3- Goodwin and Mercer, 1983; 4- Vallee and Wacker, 1970; 5- Price et al., 1972; 6- Li, 1967; 7- Randall and Bouma, 1973.

the chloroplast, stromal pH increases from 7 to 8 favoring the formation of HCO_3^- in the light. Carbonic anhydrase maintains the equilibrium so that RuBP carboxylase activity and hence CO_2 fixation is favored. Through this equilibrium, substrate is provided for carbon storage as starch or for immediate use in ATP generation via the tricarboxylic acid cycle. If HCO_3^- formation were favored, little CO_2 would be stored as starch. This would leave the plant unprotected against periods of carbon deficit. Carbonic anhydrase activity and photosynthesis are reduced and respiration is increased in Zn-deficient soybeans (Glycine max L. Merrill) (Ohki, 1978). Carbonic anhydrase also was substantially reduced in Zn deficient Citrullus vulgaris Schrad. (Sharma et al., 1981).

One of the enolases is responsible for the formation of PEP which is a pivotal high energy intermediate in carbohydrate biosynthesis. From PEP, carbohydrate biosynthesis may proceed in either a gluconeogenic or glycolytic direction.

Nucleotide pyrophosphatase is responsible for the synthesis of nucleotide diphosphates which are the precursors of GTP, CTP, UTP and ATP. Adenosine 5'-triphosphate (ATP) is the primary energy currency in organisms. The nucleotide 5'-triphosphate, ATP, would be decreased due to lack of substrate if the level of nucleotide 5'-diphosphate, ATP,is decreased. Adenosine 5'-triphosphate synthesis is found decreased in Zn deficient Alyssum bertolonii Desv. (Grossi, 1985). Sharma et al. (1981) report increased inorganic phosphate and decreased organic phosphate in Zn-deficient Citrullus vulgaris Schrad.

Phosphoglucomutase is a pivotal enzyme for gluconeogenesis versus oligo/polysaccharide synthesis. A changed rate of reaction for this enzyme could alter the normal ratio of glucose-1-phosphate to

glucose-6-phosphate. Glucose-6-phosphate is the immediate precursor for the glucose moiety which is used in sucrose biosynthesis. Glucose-1-phosphate is a precursor for the formation of the oligo/polysaccharides. A reduction in phosphoglucomutase activity would decrease the level of sucrose (a non-reducing sugar). Sharma et al. (1981) noted a marked accumulation of reducing sugars and decrease in non-reducing sugars. The above may be a possible explanation for this observation.

Nason et al. (1951) reported that the activity of tryptophan synthase was decreased in Zn-deficient Neurospora sp. It has been observed that Zn-deficient plants behave as if they are auxin deficient (Skoog, 1940). Tryptophan synthase has been shown to be involved in IAA biosynthesis (Goodwin and Mercer, 1983), as tryptorhan is the key precursor for IAA via the indole 3-pyruvic acid pathway.

In the studies of Zn-stressed Citrullus vulgaris Schrad., other noted metabolic changes were marked accumulation of non-protein nitrogen [similar to the non-protein nitrogen increase reported in blight-affected citrus (Syvertsen and Albrigo, 1984)] and a decrease in protein nitrogen and nucleic acids; and stimulated acid phosphatase and ribonuclease activities (Sharma et al., 1981). The reason for these changes may be that the activities of RNA and DNA polymerases as well as t-RNA synthetase would be reduced with Zn-deficiency. This would result in an accumulation of untranslated RNA's which would require the increased activity of ribonuclease. Nucleic acid levels and substrate for protein synthesis would be reduced, decreasing the level of protein nitrogen. There would likely be a consequent accumulation of non-protein nitrogen.

Zinc also appears necessary for the maintenance of ribosome stability and in the binding of activators and/or repressors which affect gene expression. Specifically, the species of histones present in Euglena gracilis cells grown in (-Zn) culture are quite different than when grown in (+Zn) culture. Thus Zn could be potentially growth limiting, as uncommon histones may be formed that could have a negative effect on growth potential via altered gene expression. Modification of histones by Zn appears to be through the metal-dependent methylation or phosphorylation of DNA affecting transcription or through interaction with arginine-rich peptides which inhibit activity of RNA polymerase II (Vallee, 1983). Prask and Polcke (1971) reported that cytoplasmic ribosomes of E. gracilis normally contain substantial amounts of Zn and that they become extremely unstable with Zn deficiency. Instability of ribosomes with Zn deficiency may cause an increase in RNA simply because there is no protein synthesis occurring. This too would account for a required increase in ribonuclease activity, such as that noted by Sharma et al. (1981). The reduction of RNA and ribosome cell contents is suggested as the earliest possible causal event in symptom development in the course of Zn deficiency (Price et al., 1972).

Likewise, Zn redistribution in citrus trees may cause a metabolic upset of proportions significiant enough to cause the blight syndrome. An altered Zn distribution, causing the accumulation of Zn in one tissue which affects Zn-deprivation in another tissue, would reduce the availability of the cofactor for Zn-requiring enzymes. As the above discussion emphasizes, reduction of activity of these enzymes may have significant detrimental effects on the overall metabolism of a plant.

Metal Transport and Localization

Linehan (1984) provided a model for Zn adsorption by roots and subsequent plant uptake. The model suggests that metals adsorbed on root surfaces are remobilized via organic ligands which leak from root cells. This ligand leakage is induced by the presencé of the metal ion. Lack of an external liquid diffusion path away from the root, due to the transpirational stream, prevents the accumulation of metal-ligand complexes in the free space and superficial water film of the root. Therefore the organo-metal complex is taken into the root cells and translocated along the plant axis. Thus the level of metal that is xylem transported is related to the level of metal adsorbed by their roots.

Linehan's model would accommodate the commonly used citrate model for metal binding and displacement reactions. Citrate is often used in this model due to its affinity for a diverse number of metals (Tiffin, 1972). In addition, citrate has been found to be a primary if not the primary organic ligand for Zn as well as several other metals (Chino and Baba, 1981; Godbold et al., 1984; Linehan, 1984; McGrath and Robson, 1984a; Tiffin, 1967; Tiffin, 1972; Van Goor and Wiersma, 1976). McGrath and Robson (1984a) were able to show that more Zn passed into excised stems treated with the citrate-ligand, than those treated with Zn alone. In studies of the adaptive mechanisms of plants to toxic levels of metals, Godbold et al. (1984) found that the Zn accumulation in tolerant ecotypes of Dechampsia caespitosa (L.) Beauv. as compared to non-tolerant ecotypes was correlated with the accumulation of citrate in the root sap.

Linehan suggests a concentration dependent component of metal in the soil solution that directs the level of metal moving through the plant axis. McGrath and Robson (1984b) found a similar correlation with less Zn moving up the axis of Pinus radiata D. Don under Zn deficiency conditions. In addition, they found xylary movement of Zn to be extremely slow. There was movement of Zn along the tree's axis 2 weeks after tracer application, even with sufficient levels of Zn.

The citrate model incorporates a displacement component. One report suggests a Cd-Zn antagonism (Wong et al., 1984). At low Cd levels Zn uptake is stimulated, and at high Cd levels Zn uptake is reduced. Cutler and Rains (1974) found a competitive interaction between Cd and Zn in excised barley roots.

Linehan's model and the citrate model appear to suggest passive metal uptake. In contrast, Chino and Baba (1981) suggested a requirement of metabolic energy for uptake, since they found that low temperatures and shading decreased the rate of Zn and Cd uptake as organo-metal complexes. It could be argued that these factors merely reduced the rate of transpiration thereby decreasing the rate of uptake. However, Cataldo et al. (1983) used DNP to show that the "metabolically absorbed" fraction represented 75-80% of the absorbed fraction of Cd. Zinc, Mn, Cu and Fe competed well with Cd for absorption in the study reported by Cataldo et al. (1983). This suggested the existence of a common transport site or process.

While study of the mechanism of root absorption and xylem transport is well documented, the mechanism of ion-phloem transport is not. The information available is mainly qualitative. An exception to the above, is the wealth of information concerning K^+-phloem transport. However,

this review is mainly concerned with the transport of Zn and any cations that may be related to Zn in terms of charge and size.

The phloem integrity comprising leaf, petiole, and stem traces indicates that leaf nutrients can move to other organs without extensive diversion to the xylem (Tiffin, 1972). A ^{14}C-acetate study has demonstrated assymetrical seed head development of sunflower when source leaves from one side of the stalk were removed, limiting lateral transfer of assimilates to deprived seeds (Prokofyev et al., 1957). Although much is known about macronutrient and sugar transport in the phloem, few studies deal quantitatively with micronutrient movement out of source-leaves or their form of transit.

In his review, Tiffin (1972) reports evidence of foliar applied Cu moving into new leaves and fruit; Fe into roots, or from seed to seedling; and physiological and seasonal changes in micronutrient levels of foliage (suggesting remobilization to other tissues, e.g., to developing fruit, seeds or from senescing tissues into storage tissues). When Bukovac and Riga (1962) followed Zn, P and Ca distribution from bean cotyledons to other plant parts, they found that 51% of Zn moved out of the cotyledons within 6 days of cotyledon emergence (Zn was most readily translocated). These are all suggestive of phloem involvement in metal transport.

In tracer experiments using steam girdled and non-girdled nonsenescent geranium petioles, Neumann and Chamel (1986) report of the phloem mobility of Ni, another divalent transition metal of a molecular weight similar to Zn. They demonstrated that ^{63}Ni was one-fourth as mobile as ^{86}Rb (a very mobile cation used as a K-analogue) and 25 times as mobile as Ca (a very non-mobile cation). Mengel and Kirkby (1981)

report that Ni is very phloem mobile. Zinc, Ni and Co have been detected in phloem exudates from bark incisions in stems of Ricinis communis L. (Wiersma and Van Goor, 1979). In addition, it has been demonstrated that Ni and other minerals are mobilized and transported to seed sinks via the phloem (Hocking and Pate, 1977; Misha and Kar, 1974; Neumann and Noodin, 1984). At senescence, >70% of the Ni present in soybean shoots was remobilized to seeds (Cataldo et al., 1978), >90% of which was associated with the soluble tissue fraction. With ultrafiltration of the soluble fraction >77% was shown to be in the range of 0.5-10.0 kd. Several organic complexes were resolved from the soluble fraction. Most of the remobilized Ni was associated with the cotyledons, 80% was soluble, 70% of the soluble fraction was composed of Ni-complexes with molecular weights <10kd. A soluble organic-Zn complex has been isolated in Ricinis communis L. (Van Goor and Wiersma, 1976). The molecular weight of this complex is estimated at approximately 0.5kd.

In summary, the literature suggests that Zn moves into the root via citrate through an exchange site which may be common to Cd, Mn, Cu and Fe. This uptake may be dependent on a metabolic component, although soil-Zn concentration appears important to the magnitude of Zn moving up the plant axis. This movement via the xylem appears to be very slow.

Little information is available concerning phloem transport and even less on Zn-phloem mobility. It is apparent that Zn is phloem mobile and possibly more mobile in the phloem than xylem, comparing the results of the Bukovac and Riga (1962) study with those of the McGrath and Robson (1984a;b) studies. Zinc moves through Ricinis communis L. phloem as a soluble small molecular weight complex of 0.5kd (Van Goor

and Wiersma, 1976). Similarly, Ni appears to move through the phloem as an organo-metal complex of 0.5-10.0 kd. The Ni is in a highly soluble fraction that is very mobile. Are the organo-metal complexes formed in the phloem in association with Zn and Ni related? In addition, are there similar complexes in the phloem that may be responsible for the transport of other heavy metal divalent cations?

The Metal Binding Polypeptides, Metallothionein and Phytochelatin

Recent concern over heavy metal contamination of the environment (Varma and Katz, 1978) has stimulated research on plant response to toxic levels of heavy metals (Bartolf et al., 1980; Casterline and Barnett, 1982; Girling and Peterson, 1981; Jarvis et al., 1976; Kaneta et al., 1983; Page et al., 1972; Petit and Van De Geijn, 1978; Rauser and Curvetto, 1980). Because metallothionein is an extremely effective sequestering agent of heavy metals in mammalian systems (Kagi et al., 1980), several groups have investigated a similar role in plants (Bartolf et al., 1980; Casterline and Barnett, 1982; Hardiman and Jacoby, 1984; Hogan and Rauser, 1981; Jackson et al., 1985; Wagner and Trotter, 1982).

Metallothioneins are low molecular weight cysteine-rich proteins which appear responsible for heavy metal homeostasis in animals (Kagi and Vallee, 1961; Nordberg and Kojima, 1979). The role of metallothioneins in heavy metal homeostasis involves the sequestering of toxic metals (Hg and Cd) and the regulation of essential elements such as Zn and Cu. Other functions have been proposed, including regulation of cellular metabolism and growth, detoxification of free radicals and protection against ionizing radiation (Karin, 1985).

In their review, Kagi et al. (1980) reported that these highly anionic proteins are characterized by a single polypeptide chain 61 amino acid residues in length. The chain contained 20 cysteine residues. Because they lack histidine and aromatic residues, these proteins do not show absorbance at 280 nm. Serine, lysine and alanine are also abundant in these proteins. Metallothioneins sequester the IB and IIB transition metals at distorted tetrahedral coordination sites of -cys-x-cys- in a ratio of 1:3, metal ion: cysteine residues. Metal-thiol chromophores exhibit absorbance at 254 nm. Integrity of these chromophores is reversibly lost below pH 2.0. A decrease in absorption with acidification occurs due to the breakage of the metal-mercaptide bonds (Macara, 1978). The metal-thiol complex is heat stable.

Metallothionein synthesis is induced by the presence of IB and IIB transition metals (Durnam and Palmiter, 1981). This increase is accomplished by the increased transcription of a multigene family (Karin and Richards, 1982) and in some cases by the amplification of a single gene member (Beach and Palmiter, 1981).

Recent isolation and characterization of metal binding polypeptides (phytochelatins) from higher plant cell suspension cultures has revealed that their structure and performance may differ somewhat from their functional analogues in animal systems (Grill et al., 1985; Jackson et al., 1985; Robinson et al., 1985; Wagner and Reese, personal communication, 1986; Robinson and Jackson, 1986). Although they also are cysteine-rich, these polypeptides do not contain serine, lysine and alanine, but they are rich in glutamic acid (Lolkema et al., 1984; Kondo et al., 1985; Rauser, 1984; Rauser and Glover, 1984; Webb and Cain, 1982). The peptide chain contains 7-15 amino acids of which 3-8 are cysteine.

The latest evidence indicated that phytochelatins have a polymeric structure of (γ-glutamyl-cysteine)$_n$-glycine (n= 3-8) (Grill et al., 1985; 1986; Jackson et al., 1985). The Cd-binding peptides cadystin B and cadystin A, from Schizosacchoromyces pombe, are structurally similar to the metal-binding polypeptides in plants. However, the (γ-glu-cys) unit is repeated only 2 times (n= 2). Thus the repeating unit of the metal-binding polypeptide may have a value of n= 2-8 (Kondo et al., 1985). Phytochelatins from Agrostis gigantea Roth. have a greater binding efficiency of 1:2, Cu: cysteine residue (Rauser and Curvetto, 1980) when compared to metallothionein proteins.

Presence of the γ-glu-cys linkage rather than a peptide linkage suggests that this polypeptide is not a primary gene product but rather a biosynthetic product. Enzymes involved in glutathione (γ-glutamyl-cysteinyl-glycine) synthesis may be involved in the synthesis of complexing peptides based on their structural similarities.

This class of polypeptide (phytochelatin) may bind not only IB and IIB but also transition metals from groups VIII, IVA and VA (Grill et al., 1985; 1986). Phytochelatins, like metallothioneins, also exhibit absorbance at 254 nm but lack absorbance at 280 nm. Their synthesis is metal inducible. Phytochelatins are heat stable, labile at or below pH 2 and highly anionic, like the animal polypeptides, metallothioneins.

Objectives and Justification for Study

Two questions were key to the understanding of the etiology of citrus blight, because Zn redistribution is the earliest change in symptom development of blight, and the activities of at least 2

Zn-requiring enzymes are known to be reduced at the decline stage of blight (after Zn redistribution in the predecline stage). The first is the one of primary concern in this study: is the Zn redistribution directly associated with blight and what is the mechanism of the redistribution? Second, if so, does this redistribution alter Zn metabolism and cause blight symptoms?

In this study the mechanism and the path of the Zn redistribution were sought. The literature suggests that Zn is more phloem mobile than xylem mobile (Bukovac and Riga, 1962; McGrath and Robson, 1984a;b). In addition, the phloem above the bud union is the site of Zn accumulation (Albrigo et al., 1986). Therefore, the phloem above the bud union was the primary tissue sampled in the study. There is evidence of a soluble, low molecular weight organo-metal complex in the phloem (Cataldo et al., 1978, Van Goor and Weisma, 1976). Because phytochelatins are documented as low molecular weight, heavy metal complexing agents, methods for their study were implemented, as the nature of the mechanism of Zn redistribution was sought in this study.

Early, at the predecline stage in the developmental progression of the blight syndrome, Zn accumulated in the phloem (outer trunk). However, 1-2 years later, when xylem plugging occurred at the decline stage, there was no accumulation of Zn in the plugged xylem (inner wood) (Albrigo et al., 1986; Young et al., 1980). Separation of these events in time and space suggests that Zn accumulation and xylem plugging are not directly related. Yet, the fact that the latter never takes place without first the occurrence of the former would imply a possible relationship. Therefore, study of the occurrence of a Zn complexing agent during development of the blight syndrome and with regard to

location within the tree was performed to better understand the implied relationship, if it exists. For the study of a complexing agent during blight development, phloem tissue was sampled from healthy, predecline and decline stage trees. In addition, to determine the presence of the complexing agent with regard to location, several sites were sampled within predecline stage trees.

Since Zn accumulated in one plant tissue, the trunk phloem above the bud union (Albrigo and Young, 1981), while at times it became deficient in another, the young leaves (Rhoads, 1936), the accumlation of a complexing agent in the trunk phloem was suggested. To generate excessive levels of the complexing agent, its synthesis must be induced. The nature of this induction was of interest, with regard to synthesis of a new Zn-complexing metabolite versus amplified synthesis of an existing metabolite. This was of particular interest in view of evidence of blight transmission (Tucker et al., 1984). The data of Tucker et al. (1984) data suggest the involvement of a transmmitted agent. This agent could have two possible roles. One, it may be the Zn-complexing metabolite. Two, it could induce the synthesis of the Zn-complexing metabolite.

Ultimately, information about the mechanism of the blight-induction is sought. Does the transmitted agent simply amplify transcription of a normally expressed gene or does it induce expression of a normally non-expressed portion of the genome?

Phytochelatins are regarded as normal metabolites for maintaining heavy metal homeostasis. If a Zn-complexing agent associated with blight is a phytochelatin, then induction of amplified synthesis by the transmitted agent is implied. In this event, even the phytochelatin

could be the transmitted agent. However, somewhere synthesis of the phytochelatin would have been amplified, either in a plant source or possibly in a microbe capable of phytochelatin synthesis.

The primary objective of this study was to determine if a Zn-binding molecule exists and to isolate, purify and characterize the molecule if its presence correlated with the occurrence of citrus blight. A secondary objective was to determine the primary location of the accumulated complexing agent. To determine if the Zn-complexing agent was a phytochelatin, methods for their isolation and purification and assays for their characterization were used in the study.

Isolation and purification procedures for plant and animal metal-binding polypeptides are very similar. Most laboratories working with these systems use various combinations of gel filtration, ion exchange chromatography, electrophoresis, ammonium sulfate or heat precipitation, ultrafiltration, dialysis and lyophilization (Bartolf et al., 1980; Casterline and Barnett, 1982; Grill et al., 1985; Jackson et al., 1984; Lerch, 1980; Lolkema et al., 1984; Rauser and Glover, 1984; Wagner and Trotter, 1982). Precipitation procedures are more appropriate for the larger molecular weight proteins from animal systems. Casterline and Barnett (1982) explained that such treatment resulted in some loss of Cd-containing fractions of low molecular weight in soybeans. Concentrating procedures are most useful when detectable levels of the metal-mercaptide complex are limited. The major purification procedures used are based on molecular sieving and ionic characteristics, i.e. gel filtration and ion exchange, respectively.

CHAPTER 2
MATERIALS AND METHODS

Phloem Tissue Sampling and Extraction

'Valencia' sweet orange and 'Marsh' grapefruit cultivars on rough lemon or carrizo rootstocks from 3 groves were sampled for phloem tissue from healthy, predecline and decline stages of citrus blight. Healthy, predecline and decline trees were screened and separated on the basis of Zn levels and water conductivity. Decline trees had higher Zn concentrations with reduced water conductivity. Predecline trees had an elevated Zn concentration and normal water conductivity, while healthy trees had lower Zn levels and normal water conductivity (Albrigo et al., 1986). One 10 x 10 cm bark patch was removed from each sampled tree approximately 25 cm above the bud union. The bark patch was divided into ten, 1 cm strips and transported in a plastic bag on ice from the grove to the lab. Bark strips were rinsed in deionized water and the phloem was separated from the periderm with a stainless steel blade. Ten grams (f.w.) of phloem tissue were ground for 20 minutes in a 50 ml sorvall grinder cup cooled in an ice bath. For grinding, the ten gram tissue sample was placed in approximately 25 mls of cold buffer containing 50 mM Tris-HCl, pH 7.8 with 50 mM 2-β-mercaptoethanol and 5% PVPP. The resulting homogenate was filtered through Whatman No. 1 filter paper with buffer washes up to a final volume of 30 mls/10 g phloem tissue (f.w.). The filtrate was centrifuged at 4°C for 20

minutes at 15,000 x g_{max}. The supernatant was filtered through a 0.45 µm filter and transferred to a 0.2 µm millipore filter apparatus for final filtration, in order to eliminate bacterial contamination.

Isolation and Purification of Zn-binding Factor

A 10 ml aliquot of the resulting phloem tissue extract was loaded onto a 5 x 4.1 cm column of DEAE-Sephadex A-50 for ion exchange chromatography (IEC). The column was equilibrated with 50 mM Tris-HCl, pH 7.8. A 280 ml linear gradient of either 0.25-2.0 M NaCl or 0.5-3.0 M NaCl in 50 mM Tris-HCl, pH 7.8 was applied. The flow rate was approximately 40 ml/h with either 5.0 ± 0.2 ml or 9.5 ± 0.2 ml fractions collected at 4°C. Fractions with absorbance at 254 nm and coincident Zn peaks were pooled and a 5 ml aliquot applied to a 20 x 4.1 cm column of Sephadex G-50 (fine) in the same buffer also at 4°C. The column was developed at a rate of 30 ml/h. Fractions of either 5.0 ± 0.2 ml or 9.5 ± 0.2 ml were collected. Appropriate fractions were pooled and lyophilized.

Electrophoretic Evaluation of Isolated Zn-binding Factor

Purity of isolated material was assessed on a polyacrylamide gel electrophoresis (PAGE) run in 0.15 M Tricene, pH 6.95. This gel system was specifically designed for detection of anionic, low molecular weight, metal-binding proteins from suspension cultured cabbage and tobacco cells (Reese and Wagner, personal communication, 1986). The gel was a 20% aqueous acrylamide solution (0.8% bisacrylamide) and contained 0.04% (w/v) TEMED, and 0.04% (w/v) ammonium persulfate in 1.0 M Tris-HCl, pH 7.6. Electrophoresis was performed at room temperature at a

constant current of 30 mA using 0.002% bromophenol blue as the tracking dye. Prior to electrophoresis, 45 µl of sample was mixed with 5 µl of glycerol containing 2.0% bromophenol blue in 0.15M Tricene as a sample buffer. Duplicate gels were run. One gel was sliced into 1 x 1.5 cm sections, eluted in distilled/deionized H_2O and analyzed for total Zn, A-254 nm and A-280 nm. The other gel was fixed for 5 min. in 12.5% TCA and stained for 30 min. in 0.2% coomassie blue and 12.5% TCA. This stained gel was developed in 10% TCA (Chrambach and Rodbard, 1981). Coomassie blue is a protein stain. It is unable to stain the small γ-polypeptide, phytochelatin. The absence of staining and A-280 nm activity was used as confirmation of the purity of the isolated material from classical proteins.

Characterization of Zn-binding Factor

Methods of Assay

Presence of coincident metal-thiol chromophore and Zn concentration in chromatography fractions was determined as evidence of the presence of a Zn-binding factor. Metal-thiol chromophore was assayed at A-254 nm with a Perkin-Elmer Lambda 3A UV/Vis Spectrophotometer. Total Zn was determined by atomic absorption at 213.9 nm with a Perkin-Elmer 460 Atomic Absorption Spectrophotometer.

Differential pulse polarography (DPP) is an electroanalytical technique which differentiates between free-Zn in solution (Zn^{++}) and complexed Zn (Zn^{0}) (Street and Petersen, 1978). This is in contrast to atomic absorption spectrophotometry which measures both free and complexed Zn in solution (total Zn). DPP was used to determine the capacity of each sample to reduce the level of free Zn in a 5 ppm

Zn-solution. An EG and G Princeton Applied Research Model 384B Polarographic Analyzer and the Model 303A Static Mercury Electrode were used in the assay. DPP was performed over the range of -0.800 to -1.200 V with a drop/step time of 0.6 mV/sec. Each aliquot for assay was added to a 0.1M KNO_3 matrix of 5.0 ppm Zn, pH 7.0. The 100 µl aliquot plus the 5.0 ppm Zn in KNO_3 equaled a total volume of 10 ml. The standard curve is given in Figure 1. Loss or increase in free Zn concentration in solutions containing each sample aliquot was determined and reported. (Calculations of free Zn concentration were made on the basis of the total 10 ml assay mixture.) A reduction of the concentration of free Zn was assumed to be an indication of the level of Zn complexed rather than precipitated by the sample. This assumption was made on the basis that no cloudiness (signaling precipitation) was noted during the DPP analysis; Zn is found in the phloem as an organo-metal complex (Van Goor and Wiersma, 1976); and the complex is apparently soluble since it can be extracted using an aqueous buffer and several species of a metal-binding factor are recovered after chromatographic separations. Given this assumption, complexation occurred when <5.0 ppm Zn remained in solution after the assay. The sample was considered lacking in such capacity when there was >5.0 ppm Zn in solution. That level of Zn greater than the 5.0 ppm was considered the level of intrinsic free Zn ions in the original aliquot prior to assay. Because each aliquot may have its own intrinsic concentration of complexed Zn prior to assay, any complexation reduction of free Zn that occurred would be residual. Therefore this capacity was termed residual complexation capacity, RCC. Each level of purification from crude extract through gel filtration eluate was assayed as described above, for healthy, predecline and decline stages of citrus blight.

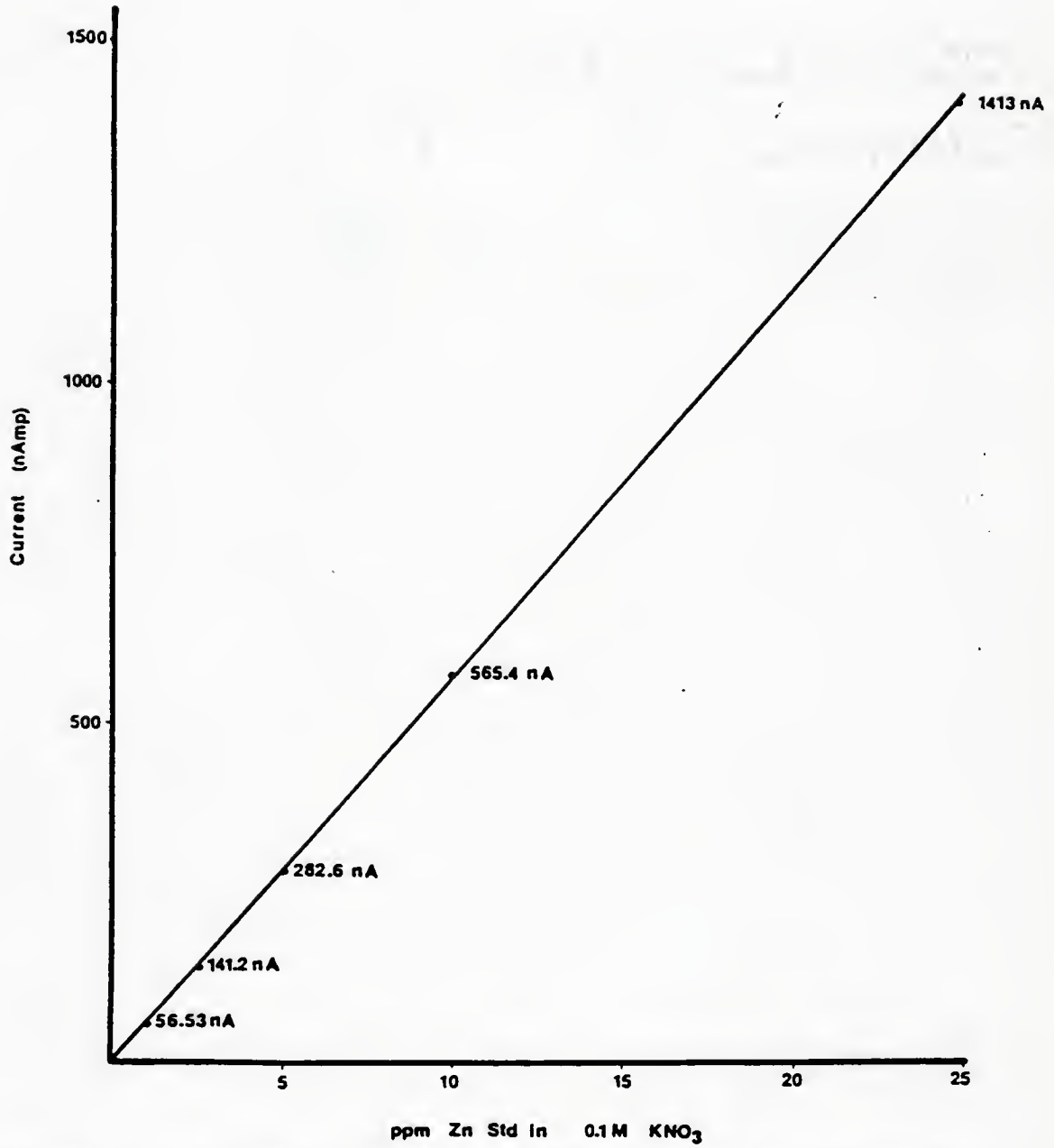


Figure 1. Calibration curve for differential pulse polarography assay.

RCC Comparisons

RCC of healthy, predecline and decline crude phloem tissue extracts were compared for their ability to complex a known amount of Pb, Cd, and Zn. The experiment was run on all extracts at varied metal concentrations of 2.5, 5.0, 7.5 and 25.0 ppm in 0.1M KNO_3. A 100 µl aliquot of each extract was contained in the 10 ml volume of extract + metal + 0.1 M KNO_3 (1% phloem extract). This experiment was performed only on 2 replicates and data are therefore considered preliminary. Upon partial purification of the crude extract via DEAE-IEC, followed by gel filtration on Sephadex G-50, 3 aliquots of predecline complexing agent were electrophoresed and four complexing species obtained. These were each assayed for RCC of Pb, Cd and Zn. Sample fractions showing A-254 nm activity and containing elevated Zn were compared and assayed.

Temperature and pH Stability

Ability to complex Zn was assayed when crude extracts of phloem tissue from decline stage trees were tested for temperature and pH stability. Prior to assay for temperature stability, sampled extracts were either incubated at room temperature (22°C) or 38°C for 2 hours, or boiled for 5 minutes. To determine pH stability, samples were adjusted to pH 1.5, 2.0, 2.8, 4.0, 6.0, 7.8, 9.0 and 10.0 and assayed for RCC. After IEC and gel filtration of a predecline crude extract, temperature was again tested. Temperatures of 20, 40, 60, 80 and 100°C were used in that portion of the experiment.

Plant Tissue Localization of Zn-Binding Factor in Predecline Trees

Each of eight tissues from 2 predecline trees was sampled for Zn binding factors: leaf and stem tissues, phloem above and below the bud

union, wood above and below the bud union, root phloem and feeder root tissues. These tissues were extracted and purified in the usual manner, except that the gel filtered material was concentrated 5-fold prior to assay. The eight tissues from the 2 predecline trees were compared for level of metal-thiol chromophore (A-254 nm), but total Zn and residual complexation capacity were determined on the eight tissues of a single predecline tree. Therefore the results from this experiment may only be considered preliminary. Values of A-254 nm, total Zn and RCC were compared to those values for the extract from healthy phloem tissue above the bud union (taken from the isolation and purification portion of the study).

CHAPTER 3
RESULTS AND DISCUSSION

Preliminary Analyses

Crude extracts from 2 decline trees were analyzed for temperature and pH effect on RCC (Figure 2). If the Zn-binding factor was not affected by temperature and pH, RCC would not be expected to change substantially from 22°C and pH 7.8. At pH's ranging from 2.8-10 there was little change in Zn complexed (range= 22.3- 24.9 ppm Zn). But with decreased pH of 2.8 to 1.5 there was a marked decrease in Zn complexation (22.3 to 1.25 ppm Zn). This reduced RCC was reversible with an increase in pH from 1.5 to 2.8 (data not shown). Temperatures ranging from 22-100°C had no effect on Zn complexed. When partially purified predecline extracts were assayed in the same manner as above for temperature effect on RCC of Zn, there was no significant change (data not shown). Over the temperature range of 100-20°C, Zn-RCC changed from 22.7 to 25.0 ppm. Classical proteins would be expected to denature and precipitate with very high temperatures, losing activity. However, this complexing agent did not. The above data suggest that this Zn-complexing agent is not a classical protein.

Crude extracts from healthy, predecline and decline tissues were placed in solutions containing 2.5, 5.0, 7.5 or 25 ppm Pb, Cd and Zn. Predecline and decline extracts apparently complexed more metal than healthy extracts. Predecline and decline extracts complexed Zn more than Cd which was complexed more than Pb (Table 2). The predecline and

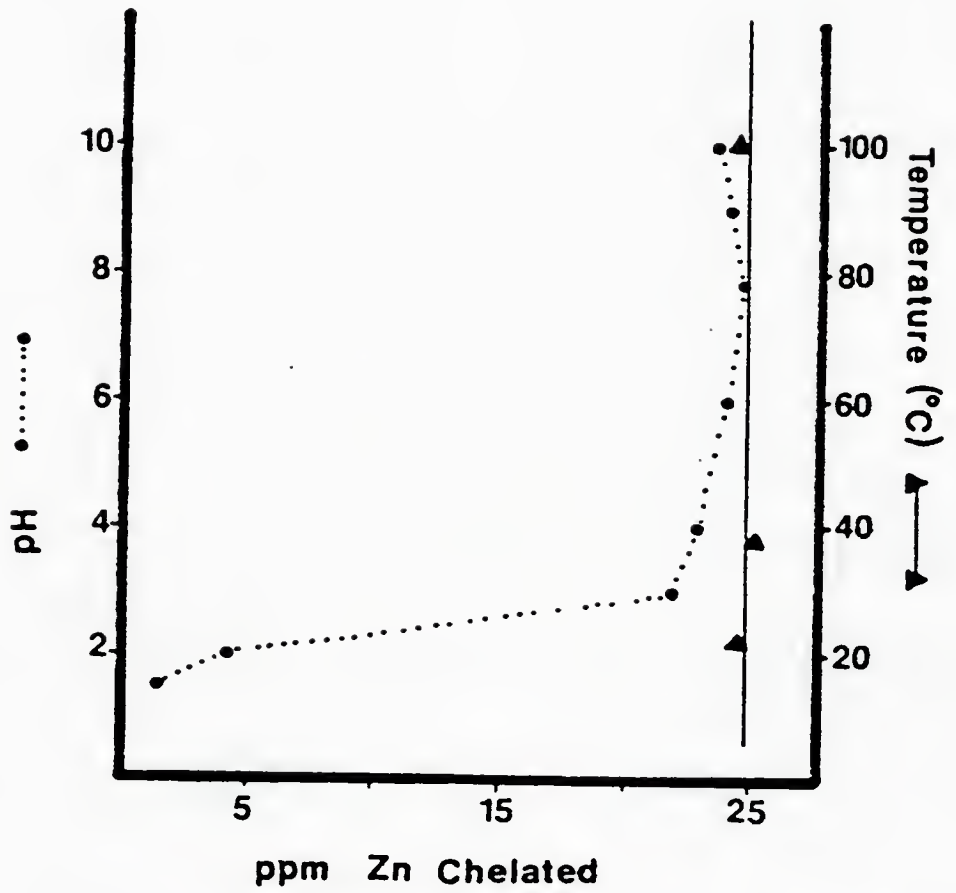


Figure 2. Effect of temperature and pH on the capacity of crude phloem tissue extract from blight-affected 'Valencia' sweet orange to complex Zn. (All data are given on the basis of extractions made on an equivalent fresh weight basis of 10 g phloem tissue/ 30 mls 50 mM Tris-HCl buffer, pH 7.8.)

Table 2.
Comparative Pb-, Cd-, and Zn-RCC by phloem extracts from healthy and blight-affected citrus trees, as assayed by DPP.[a] Samples represent activity of 100 µl of phloem tissue extract placed in 10 ml of pH 7.0, 0.1M KNO_3. (All data are given on the basis of extractions made on an equivalent fresh weight basis of 10 g phloem tissue/30 mls 50 mM Tris-HCl buffer, pH 7.8.)

Phloem Tissue Extract	ppm metal in KNO_3 solution	ppm RCC		
		Pb	Cd	Zn
Healthy	2.5	0.388	0.155	1.180
	5.0	2.029	0.505	2.206
	7.5	3.829	0.509	3.333
	25.0	5.132	0.578	4.974
Predecline	2.5	2.495	2.500	2.500
	5.0	3.312	5.998	5.000
	7.5	4.830	7.495	7.500
	25.0	18.622	20.933	25.000
Decline	2.5	1.304	2.495	2.500
	5.0	2.944	3.798	5.000
	7.5	4.515	5.178	7.500
	25.0	14.207	14.950	25.000

[a] 10 gm (f.w.) phloem/30 ml 50 mM Tris-HCl buffer, pH 7.8.

decline phloem tissue extracts appear to possess a metal-binding factor with an apparent higher affinity for Zn. This complexing factor does not appear to exist in substantial quantities in healthy phloem tissue extracts. At this stage there appeared to be a difference in the behavior of crude extracts from healthy and blight-affected trees which was significant enough to warrant further investigation of the existence of a Zn complexing agent associated with the occurrence of citrus blight.

Evidence for a Zn-binding Factor with the Occurrence of Citrus Blight

Absorbance of UV at 254 nm (A-254nm), concentration of total Zn and RCC were assayed as indicators of the presence of a Zn-binding factor. Elevated A-254 nm (which demonstrates the presence of metal-thiol chromophores) coincided with elevated total Zn concentration. This provides strong evidence that the metal-thiol chromophore is a Zn-thiol chromophore. If in addition, RCC (measure of the capacity of a sample to complex metal) coincides with A-254 nm and total Zn, further capacity of the Zn-thiol chromophore to complex Zn is demonstrated. The A-254 nm and total metal concentration assays are standard for demonstrating the presence of the metal-complexing, cysteine-rich phytochelatins. The RCC assay was added to this study to examine the surplus Zn complexing capacity of any Zn-binding factor associated with citrus blight.

DEAE-Ion Exchange Chromatography (0.25 - 2.0 M NaCl Gradient)

A-254 nm. Trunk phloem tissue extracts from healthy, predecline and decline 'Valencia' sweet orange were assayed for A-254 nm after ion

exchange chromatography (IEC) on a DEAE-Sephadex-A-50 column (elution gradient = 0.25-2.0 M NaCl). There were 4 peaks of elevated A-254 nm activity within the elution profiles (Figure 3). The healthy phloem profile contained two peaks and the predecline and decline phloem profiles contained all 4 peaks. The first two peaks, a and b (maximum values at fractions #17 and #22-24, respectively), were common to all 3 phloem extracts and there was no difference in the level of each. It may be argued that peak b is a shoulder within peak a. For reasons that will become more apparent, it is being referred to as a peak in this discussion. The next peak (c) of A-254 nm activity (maximum values at fractions #31-33) was more evident in decline stage than the predecline phloem extract. A fourth, less evident, peak of A-254 nm activity occurred at fraction 36 (point d) of the elution profiles of the predecline and decline phloem extracts. There was no difference in the quantity of this activity between these tissues.

These data demonstrate that there are 2 metal-thiol chromophores present in healthy tissue that remain in nearly equal quantity with progression to the predecline and decline stages of blight. However, two other metal-thiol chromophore species appear at the predecline and decline stages of citrus blight and are absent when citrus blight appears absent (i.e. no Zn accumulation and/or reduction in H_2O conductivity).

Total Zn. After the A-254 nm assay the above phloem extracts were assayed for total Zn (Figure 4). There were 4 coincident peaks (with regard to A-254 nm data) of elevated total Zn concentration. Again, the healthy phloem profile contained a peak around fraction #17 (peak a). This peak of elevated Zn also was contained in the predecline and

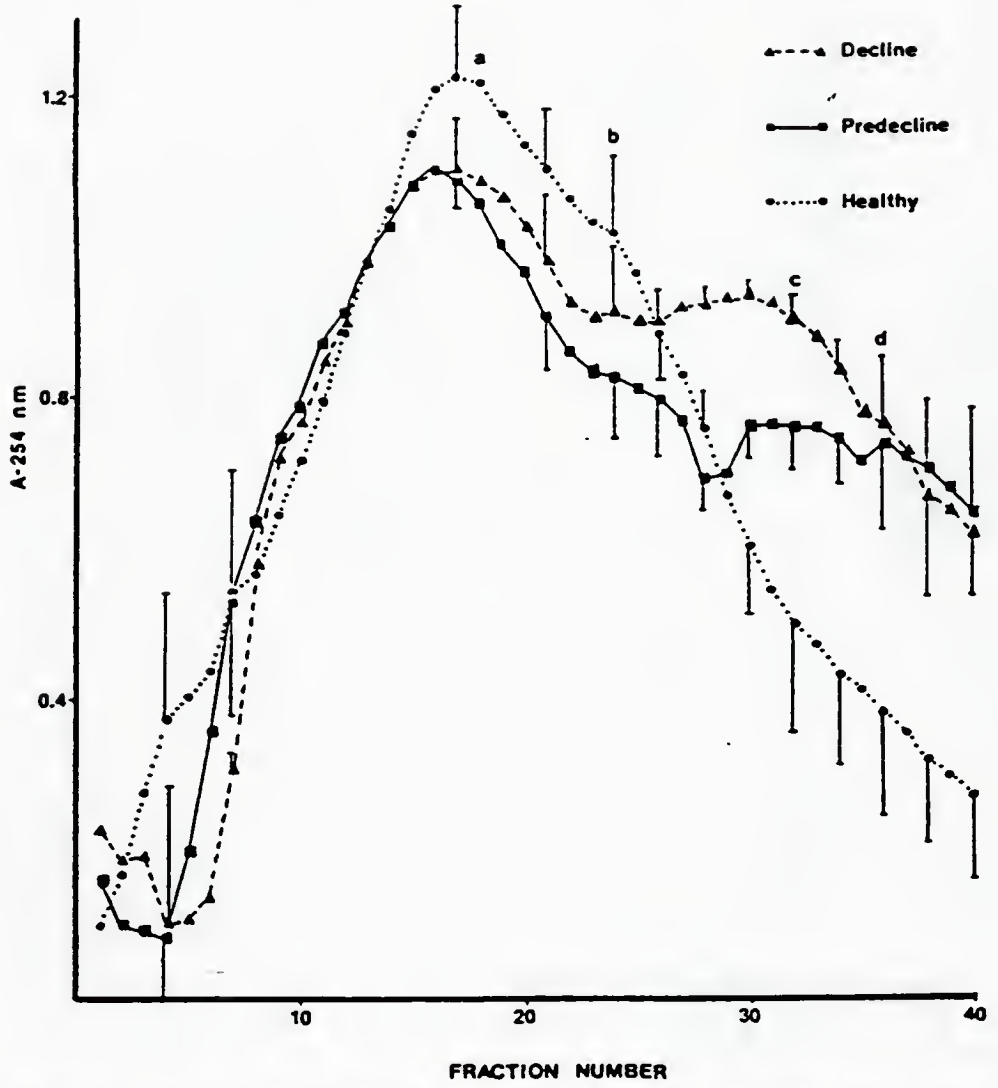


Figure 3. Absorbance at 254 nm of phloem tissue extract from healthy, predecline and decline stage, blight-affected 'Valencia' sweet orange after IEC on DEAE Sephadex-A-50 with an elution gradient of 0.25-2.0 M NaCl (a, b, c, and d represent fractions with maximum activities). (All data are given on the basis of extractions made on an equivalent fresh weight basis of 10 g phloem tissue/30 mls 50 mM Tris-HCl buffer, pH 7.8.)

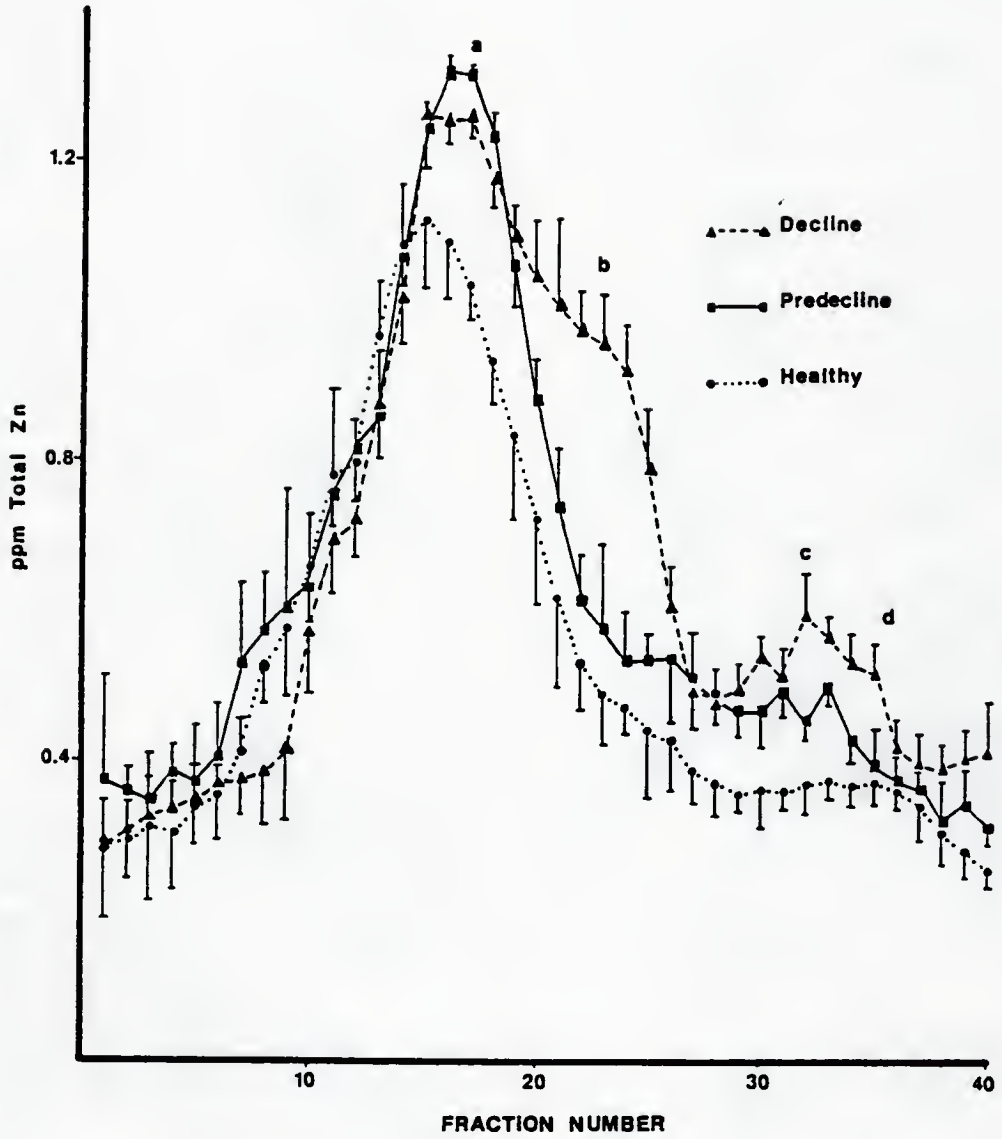


Figure 4. Concentration (ppm) of total Zn in phloem tissue extract from healthy, predecline and decline stage, blight-affected 'Valencia' sweet orange after IEC on DEAE Sephadex-A-50 with an elution gradient of 0.25-2.0 M NaCl (a, b, c, and d represent fractions with maximum activities). (All data are given on the basis of extractions made on an equivalent fresh weight basis of 10 g phloem tissue/30 mls 50 mM Tris-HCl buffer, pH 7.8.)

decline profiles with little or no difference evident among healthy, predecline and decline phloem extracts. Shouldering (peak b) was evident in the decline and almost absent in the healthy and predecline profiles. At the predecline and healthy stages, the metal-thiol chromophores indicated by peaks at b (Figure 3) may be complexes of metals other than Zn.

Peaks c and d (corresponding to fractions #30-32 and #35, respectively) were combined in the predecline and decline elution profiles and absent in the healthy elution profile. The concomitance of total Zn with A-254 nm at peak c and d in the predecline and decline elution profiles provides strong evidence of 2 Zn-thiol chromophores that were not apparent in healthy phloem extracts. This also provides evidence, by comparison (Grill et al., 1985; Jackson et al., 1985; Rauser and Glover, 1984), that the Zn-binding factors associated with citrus blight are phytochelatins. This is especially evident because those peaks of activity were strongly anionic since they require relatively concentrated NaCl for elution. Others working with phytochelatins have reported similar results (Bartolf et al, 1980; Casterline and Barnett, 1982; Rauser and Curvetto, 1980) (Table 3). The Zn-binding factors (peaks a and b) common to all phloem extracts were strongly anionic in addition to having Zn-chromophore activity. Therefore, a phytochelatin which remains even during development of the citrus blight syndrome is suggested in healthy phloem tissue.

RCC. Coincidence was only somewhat evident when the same IEC fractions were assayed for RCC (Figure 5). There was a broad area of residual Zn-complexing capacity in elution profiles of all 3 phloem tissue extracts. This area of complexation occurred at several single

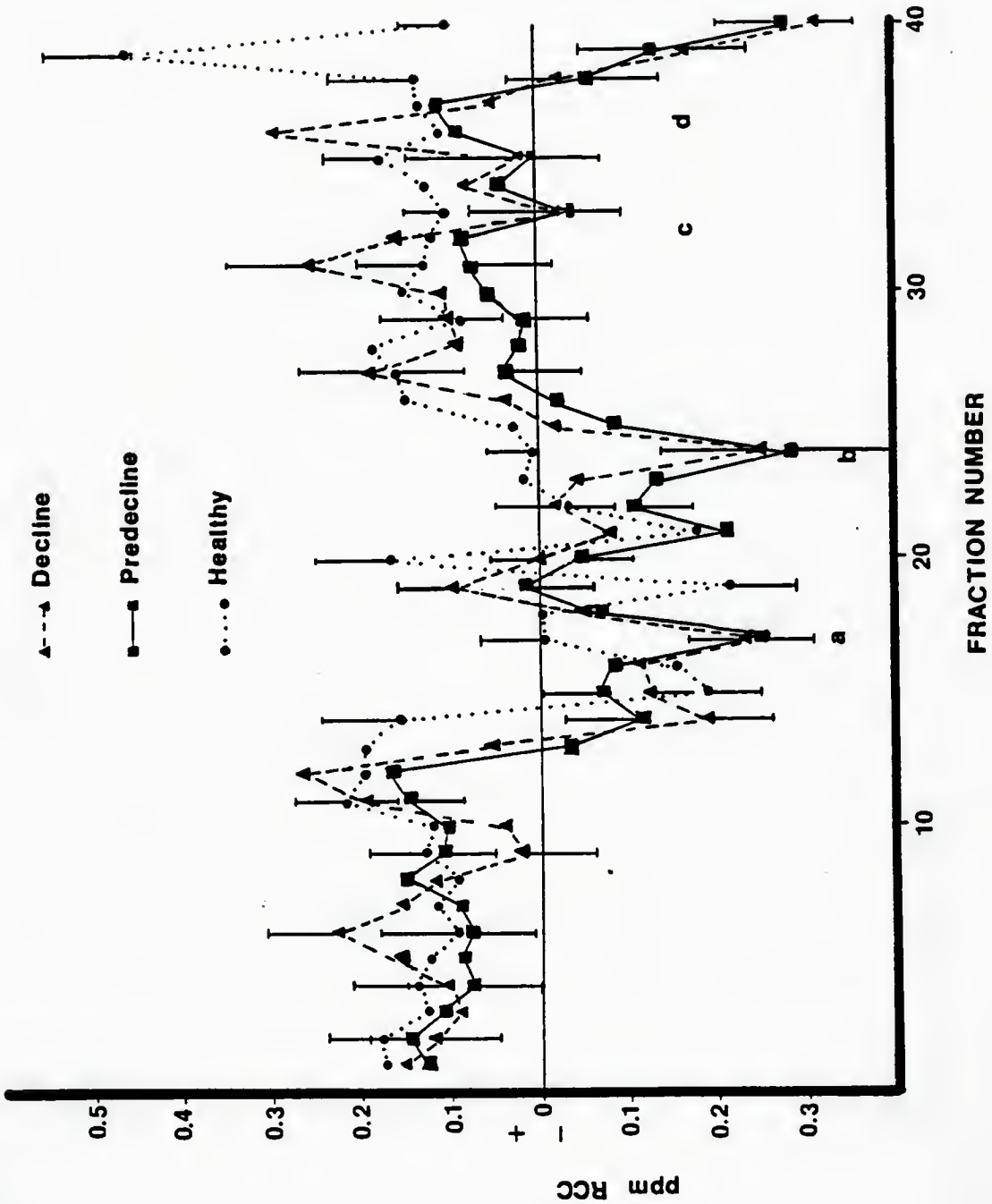
Table 3.

NaCl concentrations required to elute the corresponding Zn-binding peaks within the DEAE-IEC (0.25-2.0 M NaCl) elution profile. (All data are given on the basis of extractions made on an equivalent fresh weight basis of 10 g phloem tissue/ 30 mls 50mM Tris-HCl buffer, pH 7.8)

Peak	Concentration of NaCl (M)
a	0.60 - 0.80
b	0.97 - 1.05
c	1.45 - 1.50
d	1.55 - 1.60

fraction peaks in fractions #14-25. The corresponding areas of elevated A-254 nm activity and total Zn, encompassing points a and b were contained in fractions #10-27 and #8-24, respectively (Figures 3 and 4). Generally, there is evidence of Zn-RCC in the common region of Zn-chromophore presence in healthy, predecline and decline elution profiles. There are single fraction peaks of RCC at fractions #33 and #35 of the predecline and decline elution profiles which may correspond to peaks c and d from the A-254 nm and total Zn assays. It should be noted that fraction #35 actually shows no RCC as defined earlier. However, there is a decline in Zn concentration relative to adjacent fractions. Thus, RCC at peak d is debatable. Very significant RCC occurs at fraction #40 in decline and predecline elution profiles. This

Figure 5. RCC (ppm) of phloem tissue extract from healthy, predecline and decline stage of blight-affected 'Valencia' sweet orange after IEC on DEAE Sephadex-A-50 with an elution gradient of 0.25-2.0 M NaCl (a, b, c, and d represent fractions with maximum activities). (All data are given on the basis of extractions made on an equivalent fresh weight basis of 10 g phloem tissue/30 mls 50 mM Tris-HCl buffer, pH 7.8.)


Decline
Predecline
Healthy
0.5
0.4
0.3
0.2
0.1
+
0
−
0.1
0.2
0.3
ppm RCC
10
20
30
40
FRACTION NUMBER
a
b
c
d

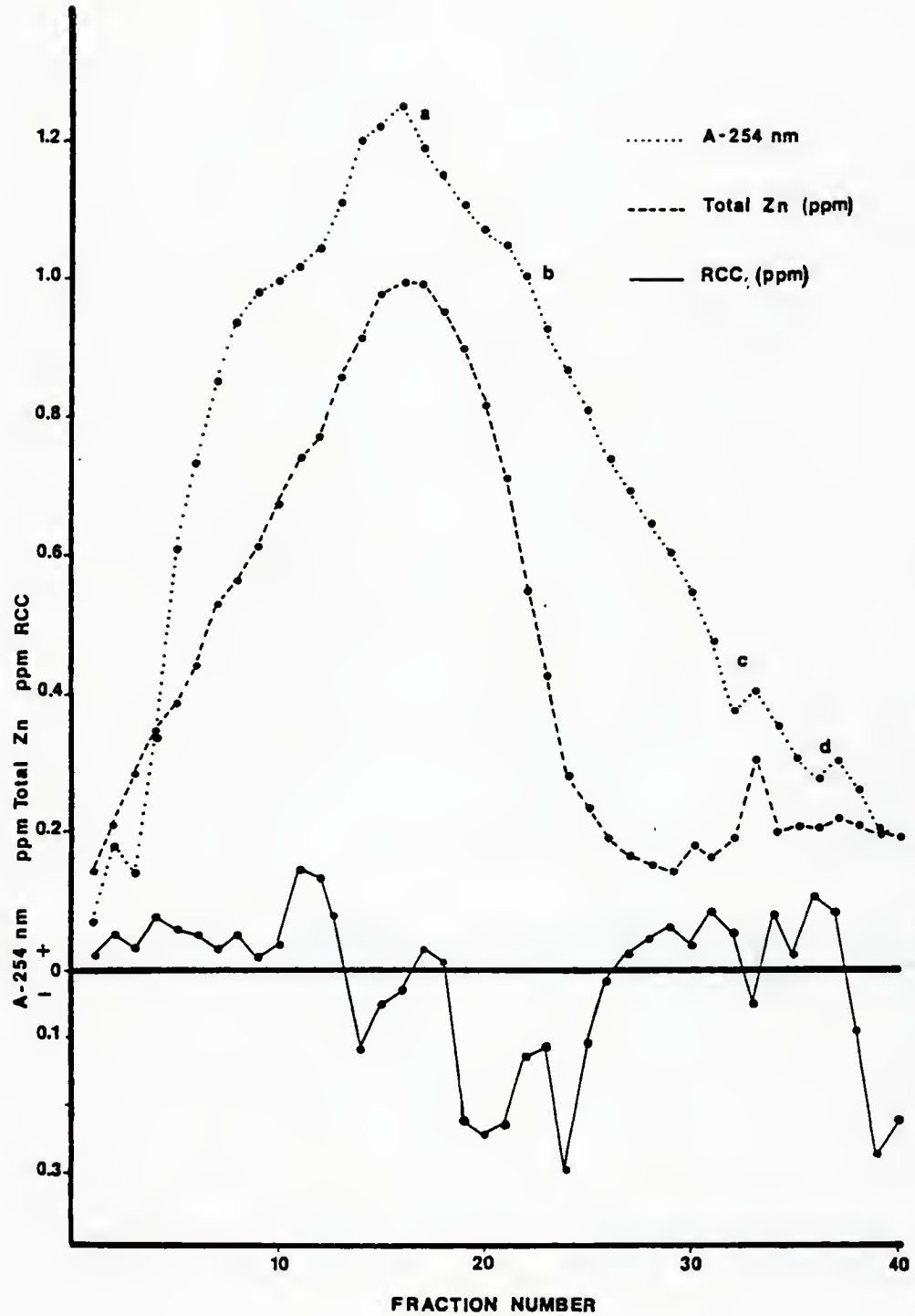
significant activity might have been due to a complexing agent which did not contain a significant level of thiolates and/or intrinsic level of Zn. There was no elevated A-254 nm activity or total Zn in the corresponding fractions for those assays. To determine if the apparent peak at fraction #40 returns to the RCC baseline, the column should be run with a somewhat extended elution gradient (possibly 0.25-2.5 M NaCl). This may effect complete elution of the peak at fraction #40. To prevent loss of resolution of the 4 other peaks and possibly increase that resolution, a greater volume of the 2 buffers composing the elution gradient should be used. This would serve to extend the number of fractions over which elution will occur, so that peaks would be clearly separate.

There appeared to be much variability within the RCC values. However, the level of variability was <5%. This appearance of variability in RCC may be characteristic of DEAE-purified material, since this variability was not apparent in material after gel filtration. A reverse order of column application (Sephadex G-50 prior to DEAE-Sephadex A-50) might be useful as a test of this hypothesis.

Absorbance at A-254 nm, Zn concentration and RCC means for 2 decline stage 'Marsh' grapefruit are given in Figure 6. The chromatography profile, though slightly different, corresponds well with that for 'Valencia' sweet orange. Peak a was centered at fraction #16, shoulder b was around fraction #22, peak c at fraction #33 and peak d at fraction #37. Again there was a Zn complexing peak of RCC at fraction #40, for which there is no clear explanation.

The concomitance of peaks a and b for orange and grapefruit (Figures 3-6) is an indication that 2 species of Zn-binding factors are

Figure 6. Comparison of A-254 nm, ppm total Zn and ppm RCC (showing coincidence in all 3 assays) in phloem tissue extract from blight-affected 'Marsh' grapefruit after IEC on DEAE Sephadex-A-50 with an elution gradient of 0.25-2.0 M NaCl (a, b, c, and d represent fractions with maximum activities). (All data are given on the basis of extractions made on an equivalent fresh weight basis of 10 g phloem tissue/30 mls 50 mM Tris-HCl buffer, pH 7.8.)


A-254 nm
Total Zn (ppm)
RCC, (ppm)
a
b
c
d
1.2
1.0
0.8
0.6
0.4
0.2
0
0.1
0.3
ppm RCC
ppm Total Zn
A-254 nm
+
−
10
20
30
40
FRACTION NUMBER

normal metabolites in the healthy citrus tree (possibly responsible for normal heavy metal homeostasis). Various assay measurements were concomitant for peak a and shoulder b in initial separations of all stages of healthy and blight-affected trees. Therefore, they were not considered in the subsequent gel filtration column series (Figures 7-8). Only those fractions which composed peaks c and d from decline and predecline tissues were pooled and gel filtered.

Gel Filtration. Values from predecline and decline samples were pooled to form the means presented in Figure 7 ('Valencia' sweet orange) and Figure 8 ('Marsh' grapefruit). Peak maximums of A-254 nm, Zn concentration and RCC were all coincident at fraction #20. An apparent molecular weight of 4 kd was determined based on a gel filtration profile which was calibrated with cytochrome c (12.3 kd), ACTH (4.5 kd) and bacitracin (1.5 kd).

DEAE-Ion Exchange Chromatoghraphy (0.5-3.0 M NaCl Gradient)

The above ion exchange series for sweet orange and grapefruit was performed using a 0.25-2.0 M NaCl gradient. To examine the molecular weight characteristics of all anionic species [peaks a, b, c and d (Figures 3-5)], another ion exchange series was performed using a steeper gradient of 0.5-3.0 M NaCl. This served to merge all anionic species (peaks a, b, c and d) into an apparent single anionic species which was placed as a single aliquot on Sephadex G-50 for gel filtration. With this approach, it could be determined whether the 4 anionic species represented one molecular weight species.

With ion exchange chromatography of healthy and blight-affected tissues of 'Valencia' sweet orange and 'Marsh' grapefruit using the 0.5-3.0 M NaCl gradient, all elevated A-254 nm activity, total Zn and

Figure 7. Comparison of A-254 nm, ppm total Zn and ppm RCC after gel filtration on Sephadex G-50 of pooled fractions #28-38 from DEAE Sephadex-A-50 (elution gradient = 0.25-2.0 M NaCl. Coincidence in all 3 assays was shown in the phloem tissue extracts from blight-affected 'Valencia' sweet orange. Zn-binding factor has an apparent molecular weight of 4kd. (All data are given on the basis of extractions made on an equivalent fresh weight basis of 10 g phloem tissue/30 mls 50 mM Tris-HCl buffer, pH 7.8.)

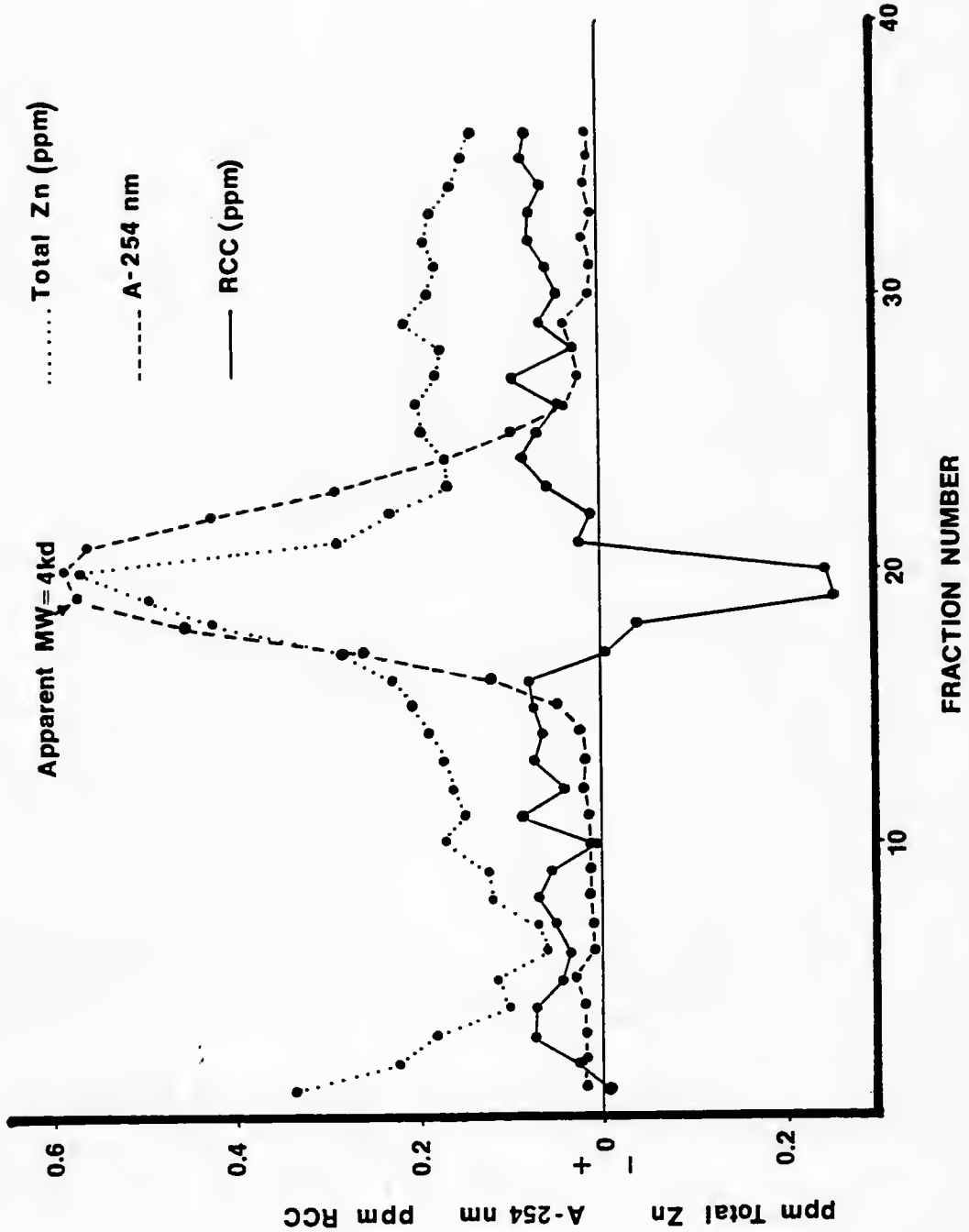
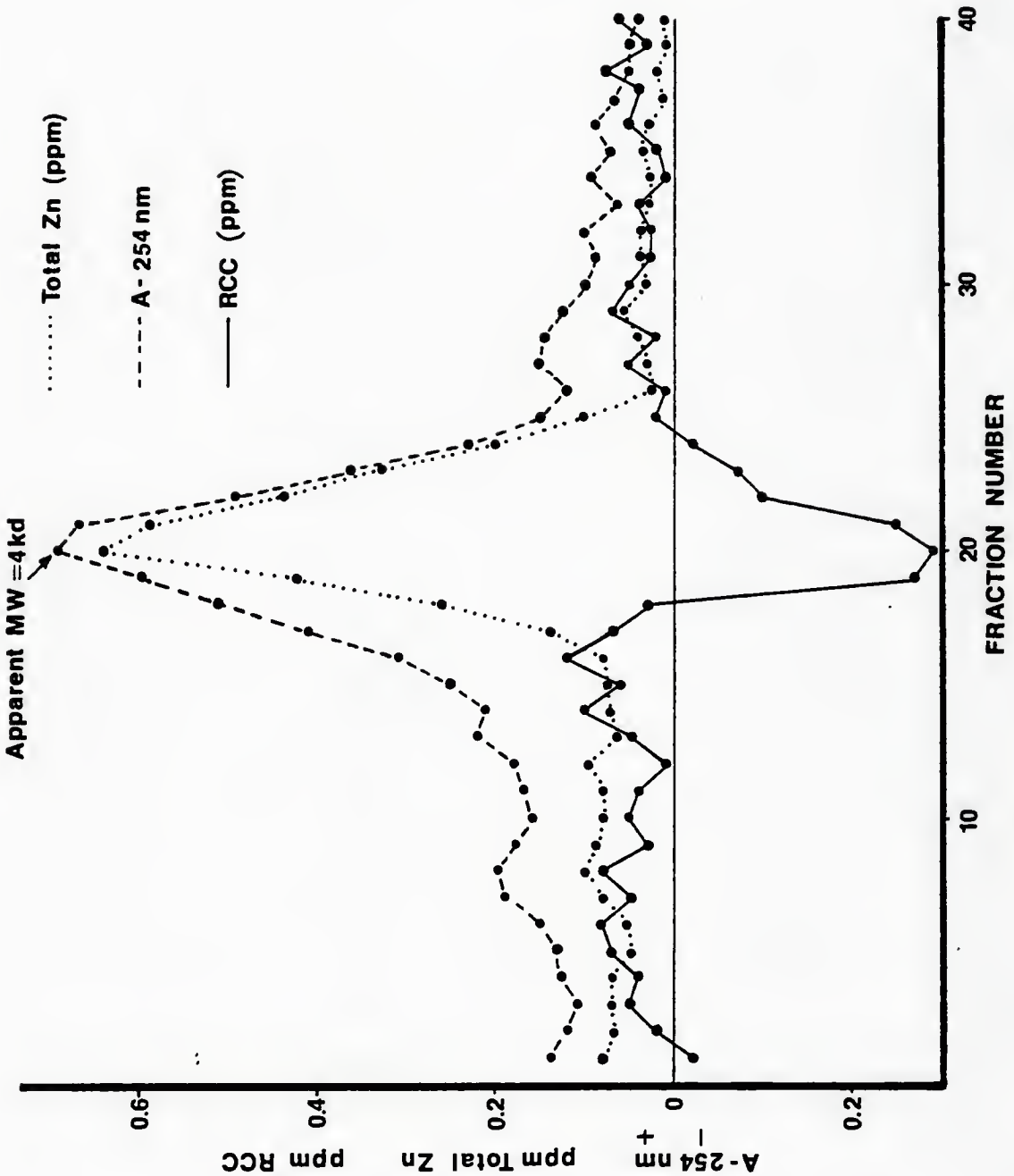

Total Zn (ppm)
A-254 nm
RCC (ppm)
Apparent MW= 4kd
0.6
0.4
0.2
0
+
-
0.2
ppm RCC
A-254 nm
ppm Total Zn
10
20
30
40
FRACTION NUMBER

Figure 8. Comparison of A-254 nm, ppm total Zn and ppm RCC (showing coincidence in all 3 assays) in phloem tissue extract from blight-affected 'Marsh' grapefruit after IEC on DEAE Sephadex-A-50 with an elution gradient of 0.25-2.0 M NaCl, with subsequent gel filtration on Sephadex G-50. Zn-binding factor has an apparent molecular weight of 4kd. (All data are given on the basis of extractions made on an equivalent fresh weight basis of 10 g phloem tissue/30 mls 50 mM Tris-HCl buffer, pH 7.8.)

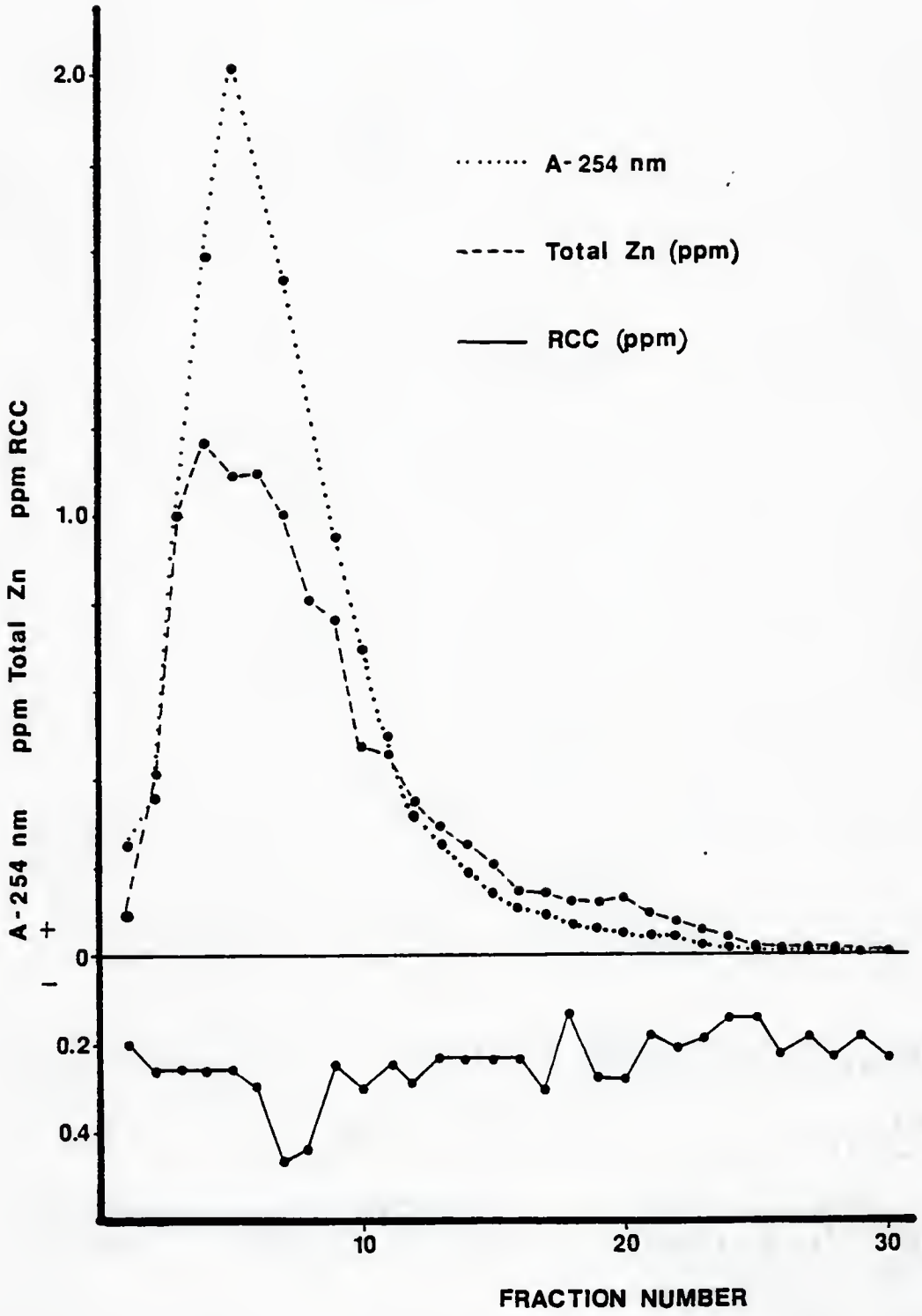

Total Zn (ppm)
A - 254 nm
RCC (ppm)
Apparent MW =4kd
0.6
0.4
0.2
0
0.2
ppm RCC
ppm Total Zn
A-254 nm
+
−
10
20
30
40
FRACTION NUMBER

RCC of Zn were merged into single peaks around fractions #5-6 (Figures 9-12). The DEAE-IEC elution profiles of sweet orange and grapefruit were very similar. However, the apparent level of Zn-binding factor detected was higher in grapefruit than sweet orange samples (Table 4). Inability to load known levels of protein (polypeptide of interest) onto the columns made it difficult to determine what the actual difference was. But sweet orange and grapefruit levels of Zn-binding factor were different when isolated, purified and assayed on the basis of equal fresh weights (Table 4).

On this basis, the 2 scions were quite different, and on a fresh weight basis, it was quite evident that the level of Zn-binding factor present in predecline trees was significantly higher than in healthy trees for both scion cultivars of citrus (Table 4). This difference is very likely due to the presence of the anionic species eluting at peaks c and d of the 0.25-2.0 M NaCl elution profile.

Gel Filtration. Fractions #2-18 from the above DEAE-IEC were pooled and an aliquot placed on the gel filtration column (Figure 13 and 14). Upon gel filtration of these IEC peaks (sweet orange and grapefruit) which contained 4 different anionic species of Zn complexing agent, an apparent single peak of Zn complexation eluted with a single apparent molecular weight of 4 kd (Figures 13-14). This however does not resolve the question of the number of molecular weight species. Within the resolving power of column chromatography (Sephadex G-50, in particular), one molecular weight species would appear likely since small differences in molecular weight would be undetected. However, Robinson et al. (1985) used an extremely long Sephadex G-50 column under dissociating conditions followed by gel filtration on low-molecular-

Figure 9. Comparison of A-254 nm, ppm total Zn and ppm RCC (showing coincidence in all 3 assays) in phloem tissue extract from blight-affected 'Valencia' sweet orange after IEC on DEAE Sephadex-A-50 with an elution gradient of 0.50-3.0 M NaCl. (All data are given on the basis of extractions made on an equivalent fresh weight basis of 10 g phloem tissue/30 mls 50 mM Tris-HCl buffer, pH 7.8.)


2.0
1.0
0
0.2
0.4
A-254 nm
Total Zn (ppm)
RCC (ppm)
A-254 nm ppm Total Zn ppm RCC
+
-
10
20
30
FRACTION NUMBER

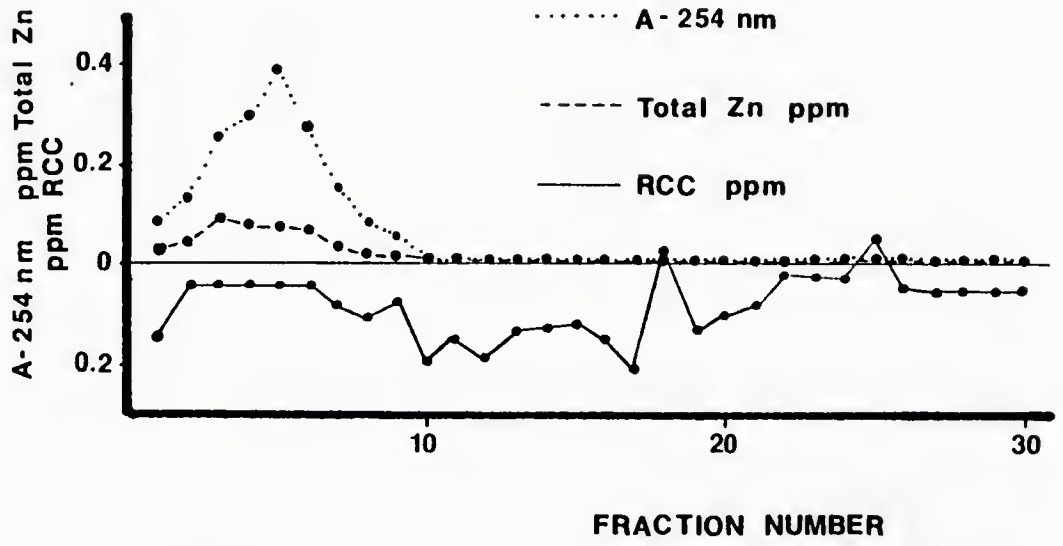
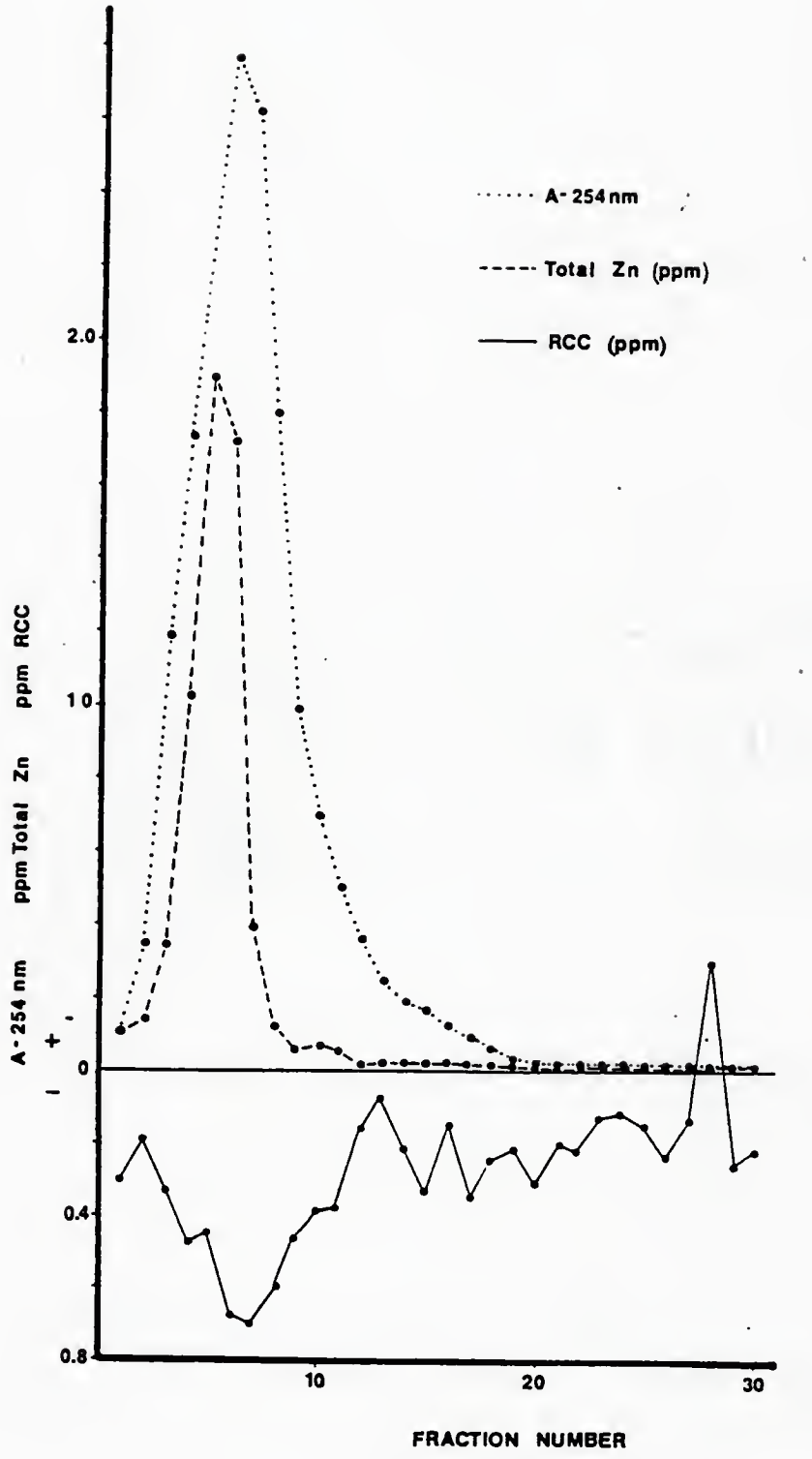


Figure 10. Comparison of A-254 nm, ppm total Zn and ppm RCC (showing coincidence in all 3 assays) in phloem tissue extract from healthy 'Valencia' sweet orange after IEC on DEAE Sephadex-A-50 with an elution gradient of 0.50-3.0 M NaCl. (All data are given on the basis of extractions made on an equivalent fresh weight basis of 10 g phloem tissue/30 mls 50 mM Tris-HCl buffer, pH 7.8.)

Figure 11. Comparison of A-254 nm, ppm total Zn and ppm RCC (showing coincidence in all 3 assays) in phloem tissue extract from blight-affected 'Marsh' grapefruit after IEC on DEAE Sephadex-A-50 with an elution gradient of 0.50-3.0 M NaCl. (All data are given on the basis of extractions made on an equivalent fresh weight basis of 10 g phloem tissue/30 mls 50 mM Tris-HCl buffer, pH 7.8.)


A-254nm
Total Zn (ppm)
RCC (ppm)
A-254 nm ppm Total Zn ppm RCC
2.0
1.0
0
0.4
0.8
10
20
30
FRACTION NUMBER

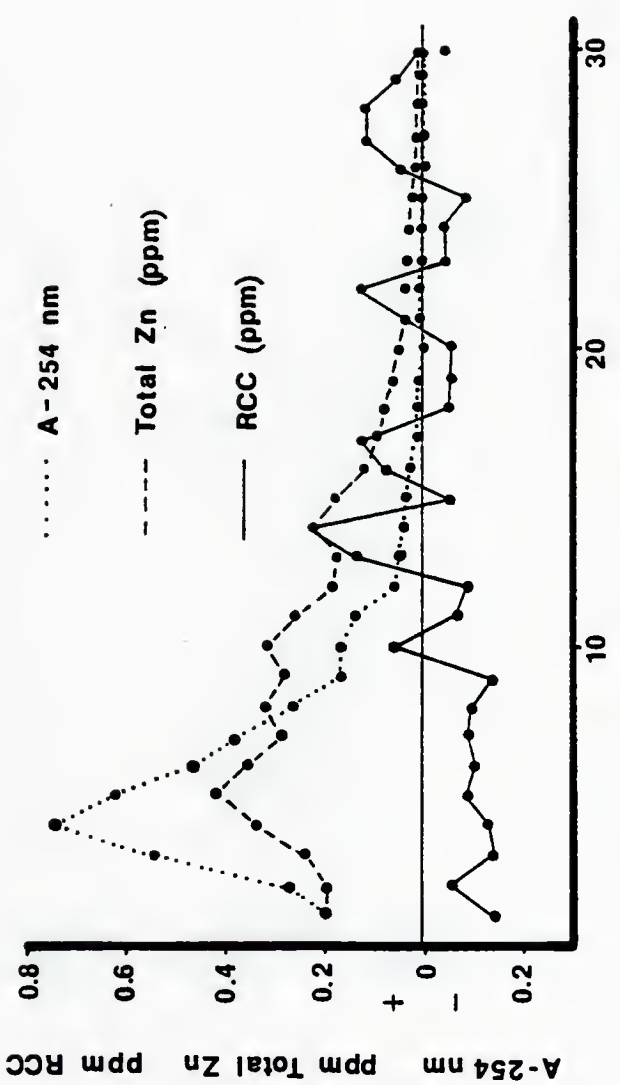


Figure 12. Comparison of A-254 nm, ppm total Zn and ppm RCC (showing coincidence in all 3 assays) in phloem tissue extractfrom healthy 'Marsh' grapefruit after IEC on DEAE Sephadex-A-50 with an elution gradient of 0.50-3.0 M NaCl. (All data are given on the basis of extractions made on an equivalent fresh weight basis of 10 g phloem tissue/30 mls 50 mM Tris-HCl buffer, pH 7.8.)

Table 4.
Comparison of the maximum levels of A-254 nm, ppm Total Zn and ppm RCC in the composite gel filtered material (from 0.50-3.0 M NaCl DEAE-IEC) in 'Valencia' sweet orange and 'Marsh' grapefruit.
(All data are given on the basis of extractions made on an equivalent fresh weight basis of 10 g phloem tissue/30 mls 50 mM Tris-HCl buffer, pH 7.8.)

Tissue	Cultivar		A-254 nm	ppm Total Zn	ppm RCC	ppm TCC[c]	A-254nm/ ppm TCC
Healthy	'Valencia'	(3)[a]	0.392 [0.109][b]	0.076 [0.066]	0.040 [0.028]	0.116	3.379
	'Marsh	(2)	0.749 [0.183]	0.418 [0.205]	0.126 [0.052]	0.544	1.377
Predecline	'Valencia'	(3)	2.014 [0.319]	1.168 [0.637]	0.465 [0.185]	1.633	1.233
	'Marsh'	(2)	2.718 [0.385]	2.892 [0.824]	0.697 [0.271]	3.589	0.757

[a]Number of replicates is given in parenthesis.
[b]Standard deviation is given in brackets.
[c]TCC = ppm Total Zn + ppm RCC.

Figure 13. Comparison of A-254 nm, ppm total Zn and ppm RCC after gel filtration on Sephadex G-50 of pooled fractions 2-13 from DEAE Sephadex-A-50 (elution gradient = 0.50-3.0 M NaCl). Coincidence in all 3 assays was shown in the phloem tissue extracts from blight-affected 'Valencia' sweet orange. Zn-binding factor has an apparent molecular weight of 4kd. (All data are given on the basis of extractions made on an equivalent fresh weight basis of 10 g phloem tissue/30 mls 50 mM Tris-HCl buffer, pH 7.8.)

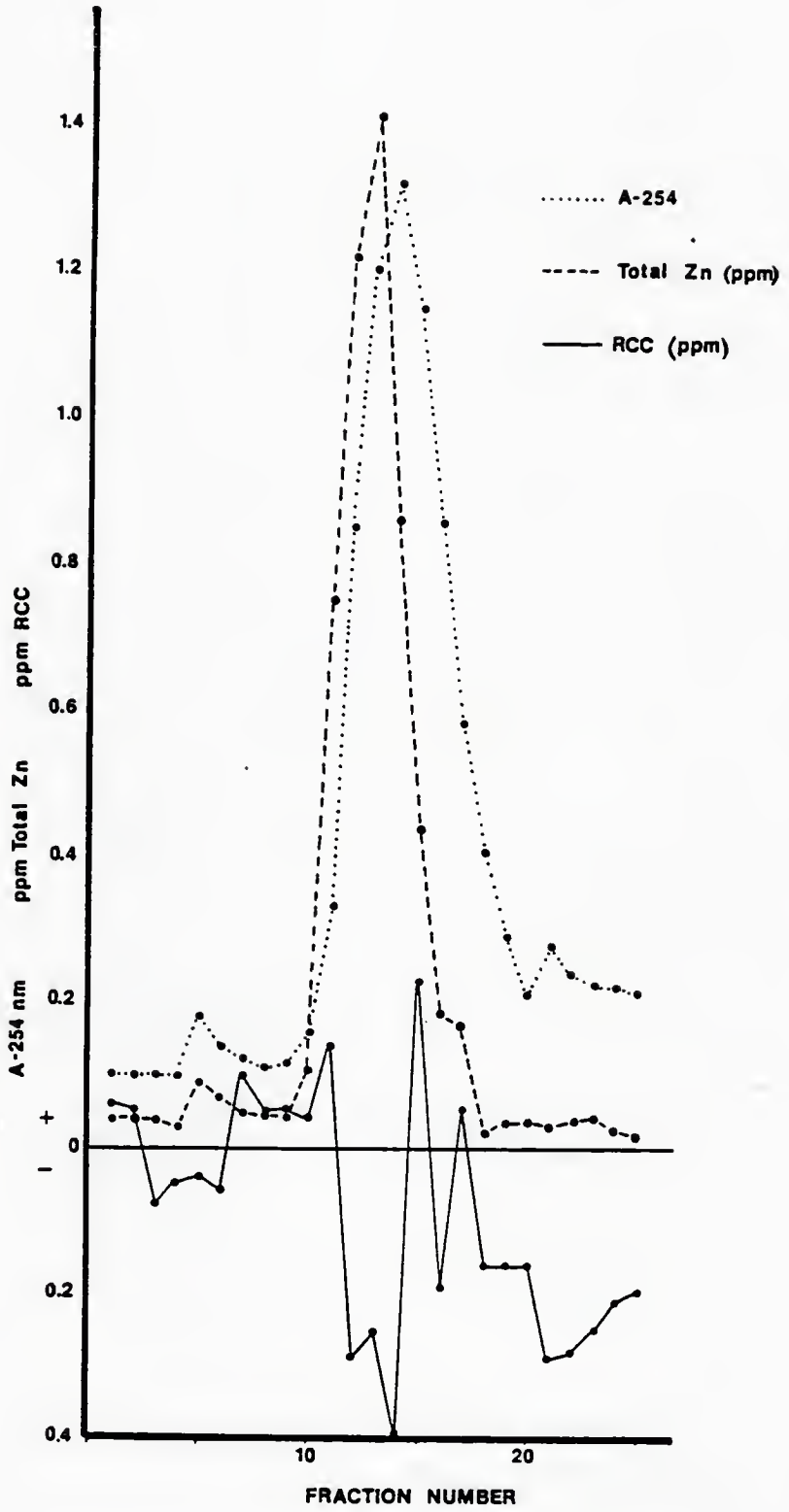
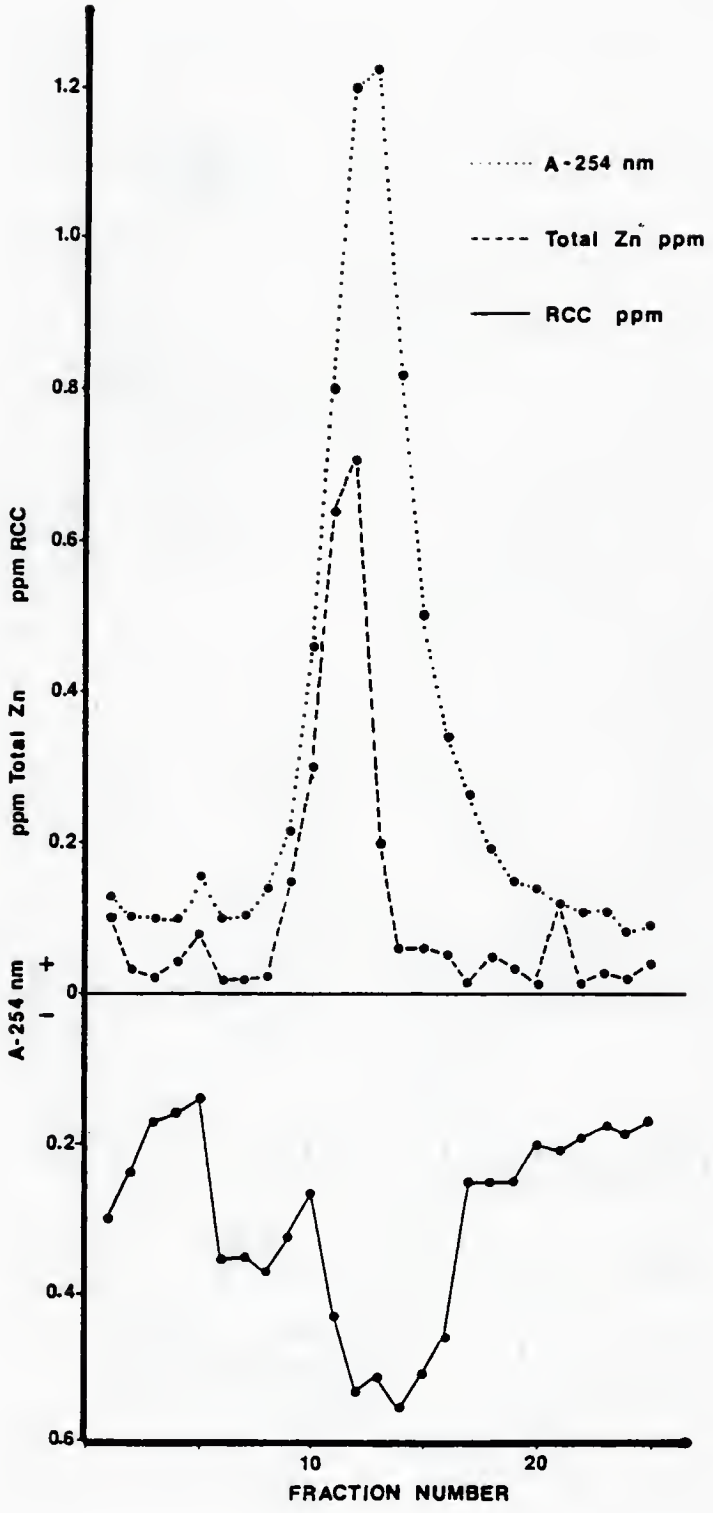

A-254
Total Zn (ppm)
RCC (ppm)
A-254 nm
ppm Total Zn
ppm RCC
1.4
1.2
1.0
0.8
0.6
0.4
0.2
+
0
−
0.2
0.4
10
20
FRACTION NUMBER

Figure 14. Comparison of A-254 nm, ppm total Zn and ppm RCC after gel filtration on Sephadex G-50 of pooled fractions 2-13 from DEAE Sephadex-A-50 (elution gradient = 0.50-3.0 M NaCl). Coincidence in all 3 assays was shown in the phloem tissue extracts from blight-affected 'Marsh' grapefruit. Zn-binding factor has an apparent molecular weight of 4kd. (All data are given on the basis of extractions made on an equivalent fresh weight basis of 10 g phloem tissue/30 mls 50 mM Tris-HCl buffer, pH 7.8.)


A-254 nm
Total Zn ppm
RCC ppm
ppm RCC
ppm Total Zn
A-254 nm
1.2
1.0
0.8
0.6
0.4
0.2
0
+
-
0.2
0.4
0.6
10
20
FRACTION NUMBER

weight selectivity columns, resolving 2 peptides with approximate molecular weights of 600-800 from Datura inoxia, Mill. This gel filtration was performed in the presence of the denaturant guanidine HCl and yielded molecular weight data in agreement with amino acid sequence data that had suggested the presence of Cd-binding multimers. Based on reports of those working with phytochelatins, the molecular weight of the denatured form of Zn-complexing agent associated with blight would be lower than 4kd (Grill et al., 1985; Jackson, personal communication, 1985). The denaturant removes metal ions and associated H_2O molecules, leaving only the amino acids. The higher level of resolution obtainable with HPLC may allow a distinction between molecular weight species. Thus HPLC may be an appropriate separation method for this application.

The finding of several metal binding species is not unprecedented. Jackson et al. (personal communication, 1986) have found that different species of $(\gamma\text{-glu-cys})_n\text{-gly}$ polymer from Datura inoxia Mill. have preference for different heavy metals. Using ^{13}C-NMR, they have demonstrated that the n= 2,3 species of the phytochelatin preferentially binds Cu, while the n= 5,7 species binds Cd. Jackson et al. (1985) have suggested that the n= 9 species may bind Zn. Grill et al. (1986) have reported similar findings for these and other metals. All forms appear capable of binding other metals, though they prefer one metal ion over the others. Of the 4 apparent species isolated in predecline trees, one or more may bind Zn, with certain species having more or less preference for Zn. If the Zn-complexing agents are phytochelatins, the synthesis of all 4 could be induced by the same agent. The n=3 and n=5 forms of phytochelatin reported by Robinson et al. (1986) were induced by Cu and Cd, respectively. They suggested the multiple forms were biosynthetic

products of a single enzyme of glutathione synthesis, γ-glutamyl transpeptidase. This enzyme may be responsible for the assembly of (γ-glutamyl-cysteinyl)$_n$-glycine. It has been hypothesized that it uses glutathione and an activated form of γ-glu-cys as precursors.

$$\gamma\text{-glu-cys-gly} + \gamma\text{-glu-cys} \xrightarrow{(\gamma\text{-glytamyl transpeptidase})} (\gamma\text{-glu-cys})_2\text{-gly}$$

Binding of Cd to (γ-glu-cys)$_n$-gly elicits product removal. Increased synthesis of the peptides in response to Cd is accompanied by increased production of certain mRNA species (Robinson et al., 1986). This may indicate amplification of normally expressed portion(s) of the genome.

To address the question of whether the Zn-binding factor is a normal or new metabolite, it is first assumed that this complexing agent is a phytochelatin. This assumption is based on the coincidence of the 3 assays which indicate the presence of the Zn-binding factors. This coincidence has been demonstrated by others as evidence of phytochelatins (Rauser and Curvetto, 1980; Grill et al., 1985; Jackson et al., 1985). The low molecular weight of the Zn-binding factors, their highly anionic character and their heat stability also add credence to this assumption. It then follows from the above that synthesis of more of a normal metabolite is being induced and that either extended or truncated forms of the (γ-glu-cys)$_n$-gly may be synthesized as well to give the additional 2 species found in the blight tree. These forms may vary in anionic character simply because there are more or less of the cysteine residues which provide the (-) charges for the binding of Zn^{++}. Their addition would cause small differences in molecular weight of approximately 268, exclusive of any additional Zn bound to the new species. It may be significant that the new species

noted in blight-affected trees are more highly anionic, and that a possible fifth species, at fraction #40 (DEAE-IEC = 0.25-2.0 M NaCl gradient) is extremely anionic. As new species appear with citrus blight progression, there may be more extended forms of those existing in healthy trees (species-peaks a and b). This is reasonable since the "fifth species" at fraction #40 showed no A-254 nm activity or elevated total Zn but showed a very high Zn-RCC. In this case the $(\gamma\text{-glu-cys})_n\text{-gly}$ may be present, but may not yet be associated with a metal. In the RCC assay, an aliquot of Zn was added to the test solution (to form 10 ml of test solution at 5 ppm Zn). At that point a metal was available to form the metal-thiol chromophore (in the RCC assay, there was a reduction of Zn in solution from 5 ppm). This data would have been more conclusive if a follow-up A-254 nm assay had been performed. As a further investigation of this phenomenon it would be helpful to treat the individual species with saturation levels of Zn, dialyze them to remove the excess Zn and then assay to see if their anionic character is altered (are the same species present?) and assay for their behavior regarding A-254 nm, total Zn and RCC. It is certainly possible that the various species only differ in the amount of Zn they have sequestered. They may appear anionically different on this basis. The Zn-saturation experiment outlined above may allow a distinction between possible forms.

Correlation of A-254 nm, Total Zn and RCC

Because A-254 nm, total Zn and RCC assays had a high degree of coincidence at points a, b, c and d, a correlation analysis was performed (Table 5). This analysis considered gel filtration data (pooled fractions #27-38) from the 0.25-2.0 M NaCl column series or

(pooled fractions #2-13) from the 0.50-3.0 M NaCl column series. In order to correlate the 3 assays used to show the presence of the Zn-binding factor, A-254 nm was compared to the sum of total Zn and RCC, yielding total complexation capacity (TCC). TCC represents the total Zn in a given fraction + the additional Zn it is capable of binding as determine using DPP. The comparisons were made in tissue extracts after gel filtration (Table 5). All were found to be significant at $p>5$ or $p<10$. The lack of correlation at $p<5$ may be due to the high variability found from sample to sample. This may be reduced if a constant quantity of the Zn complexing agent can be loaded onto the chromatography columns. As yet, a reliable assay for quantification of phytochelatin is unknown. The standard protein assays will not work because of the small size of the complexing agent, the γ-carboxyl linkage or the lack of histidine and aromatic residues. Ninhydrin which quantifies the terminal amino groups in a sample (Jackson, personal communication, 1985) or Ellman's reagent which quantifies the level of thiol groups

Table 5.
R values for A-254 nm and TCC correlation. (All data are given on the basis of extractions made on an equivalent fresh weight basis of 10 g phloem tissue/30 mls 50mM Tris-HCl buffer, pH 7.8)

Column Series	R Values
0.25-2.0 M NaCl	0.6017 (9)[a]
0.50-3.0 M NaCl	0.5873 (10)

[a]Values in parenthesis represent the number of replicates considered in the correlation.

(Ellman, 1959) may offer possible quantification procedures. In this study, samples were applied to the columns on the basis of fresh weight of active phloem.

On the other hand the level of correlation may be as much as one could expect. With reference to Table 4, study of the relationship of A-254 nm with TCC at the peak maximum (given as a ratio of A-254 nm/TCC) would suggest that no relationship should exist. Apparently, the integration achieved by the pairwise comparison of A-254 nm and TCC values of each fraction along the elution profile was adequate to give the levels of correlation given in Table 5. The analyses were performed on 9 pairs of data from the DEAE-IEC (0.25-2.0 M NaCl) and 10 pairs from the DEAE-IEC (0.50-3.0 M NaCl).

Electrophoretic Evaluation of Zn-Binding Factor

The Zn-binding factors isolated by ion exchange and gel filtration chromatography of 4 predecline phloem tissue extracts were found essentially free of classical protein contaminants when stained with coomassie-blue after PAGE. Newly purified sample immediately electrophoresed was found to be free of contaminants. A small amount of contamination was found in 2 lanes in samples stored for several weeks at 4°C. This may have been due to microbial growth. Under the coomassie-blue stain, the Zn complexing agent is free of any stainable activity. Verification of this may be achieved with the more sensitive silver stain (Chrambach and Rodbard, 1981), or with ninhydrin staining of denatured material (Jackson, personal communication, 1985).

Two gels were run in duplicate. Non-stained gels (representing 4 purified samples) were sectioned, eluted and assayed for A-254 nm, A-280 nm (Figure 15) and total Zn (Figure 16). The A-280 nm data provided

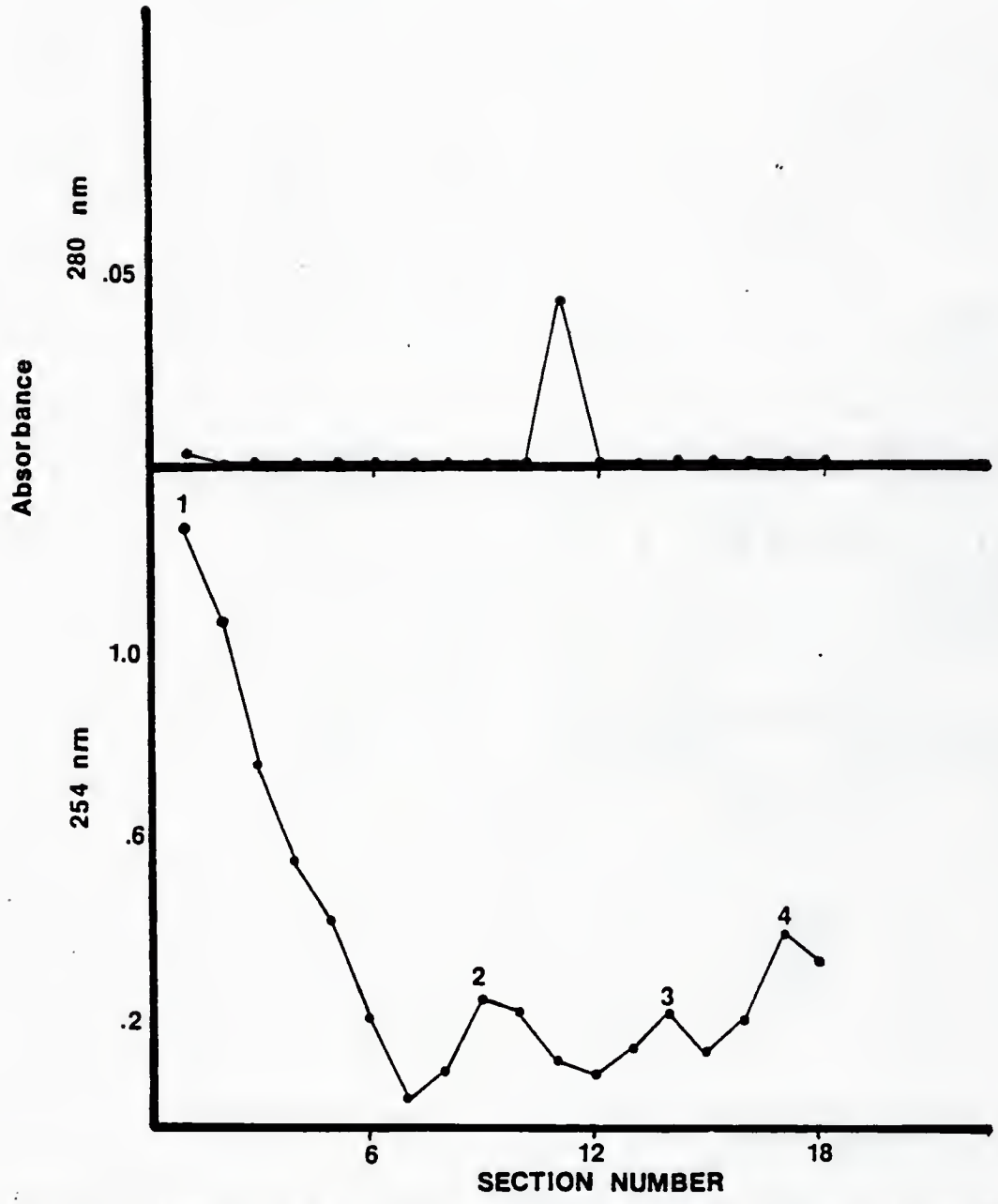


Figure 15. Absorbance at 254 nm and 280 nm of gel filtration pooled fractions #9-20 after polyacrylamide gel electrophoresis. The predecline stage, blight-affected 'Valencia' sweet orange phloem tissue extract was partially purified on DEAE-IEC (elution gradient 0.50-3.0M NaCl) with subsequent gel filtration on Sephadex G-50 (1-4 represent species peaks isolated from PAGE). (All data are given on the basis of extractions made on an equivalent fresh weight basis of 10 g phloem tissue/30 mls 50 mM Tris-HCl buffer, pH 7.8.)

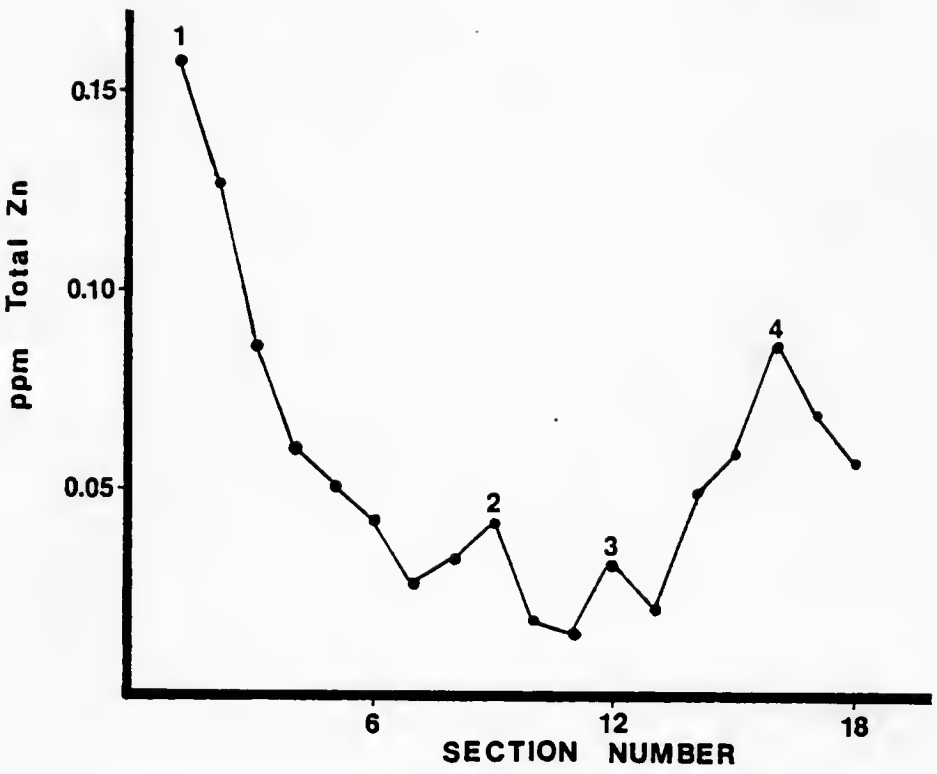


Figure 16. Concentration (ppm) of total Zn in gel filtration pooled in fractions #9-20 after polyacrylamide gel electrophoresis. The predecline stage, blight-affected 'Valencia' sweet orange phloem tissue extract was partially purified on DEAE-IEC (elution gradient = 0.50-3.0 M NaCl), with subsequent gel filtration on Sephadex G-50 (1-4 represent species peaks isolated from PAGE). (All data are given on the basis of extractions made on an equivalent fresh weight basis of 10 g phloem tissue/30 mls 50 mM Tris-HCl buffer, pH 7.8.)

further evidence that the isolated complexing agents were free of protein contamination. Findings, based on the use of a native gel system which operated on the basis of charge, indicated the presence of 4 anionic species. Coinciding peaks of A-254 nm and total Zn were apparent at sections 1, 9, 14 and 16 of the sectioned gels (Figures 15 and 16). These data contribute further evidence to that from the DEAE-IEC run with 0.25-2.0 M NaCl gradient which demonstrated the presence of 4 species. Since the sample preparation prior to electrophoresis involved the use of the 0.5-3.0 M NaCl gradient, it is not certain that the peak apparent at fraction #40 of the RCC data (from the DEAE-IEC run with 0.25-2.0 M NaCl gradient) was included as part of the sample placed on the gel. This gel system was not useful for determination of molecular weight. As yet, no one working with the small molecular weight (non-aggregated) phytochelatins has been able to make a molecular weight determination based on electrophoretic data.

An experiment was performed comparing the relative abilities of the 4 electrophoretically separated anionic species to complex Pb, Cd and Zn. The 4 anionic species were assessed separately (Table 6). The test-solution contained 25 ppm each of Pb, Cd and Zn. The data indicated that the different species have varied abilities to complex the three cations. The first two anionic species, represented by the data for section-peaks 1 and 2, complexed more Zn and Cd than Pb. Of Zn and Cd, Zn was complexed in the greatest quantity. This is reasonable, since these species, being the least anionic of the 4, probably correspond to the species found in healthy phloem tissue from DEAE-IEC data. It is also interesting that species-peak 2 which may correspond to peak b from DEAE-IEC (0.50-3.0 M NaCl) contained the greatest overall

Table 6.
Comparative Pb-, Cd-, and Zn-RCC by purified Zn-binding factor from predecline tissue extract, as assayed by DPP. Samples represent activity of 100 µl of purified material[a] placed in 10 ml of pH 7.0, 0.1M KNO_3. DPP was performed with a 25 ppm Pb, Cd, and Zn solution. (All data are given on the basis of extractions made on an equivalent fresh weight basis of 10 g phloem tissue/30 mls 50 mM Tris-HCl buffer, pH 7.8.)

Species-Peaks	ppm RCC		
	Pb	Cd	Zn
1	0.00	0.64	0.89
2	1.57	2.21	2.73
3	1.34	0.89	0.36
4	1.07	1.24	0.33

[a]Pooled fractions #2-13 from DEAE-IEC (0.5-3.0 M NaCl gradient) were placed on Sephadex G-50 for gel filtration. Subsequent pooled fractions #9-20 were separated by PAGE to yield species 1-4 which were assayed for Pb-, Cd- and Zn-RCC.

RCC. This peak had very little total Zn (Figure 4). Possibly this species had very high potential metal-thiol chromophore content, possessing a large quantity of non-complexed metal-binding factor. The species represented by peak 3 sequestered Pb>Cd>Zn and peak 4 Cd>Pb>Zn. The latter peaks appear to have an affinity for different ions, as compared to the 2 former peaks. This might be expected as they are probably somewhat different forms. These are probably extended forms of those existing in healthy tissue.

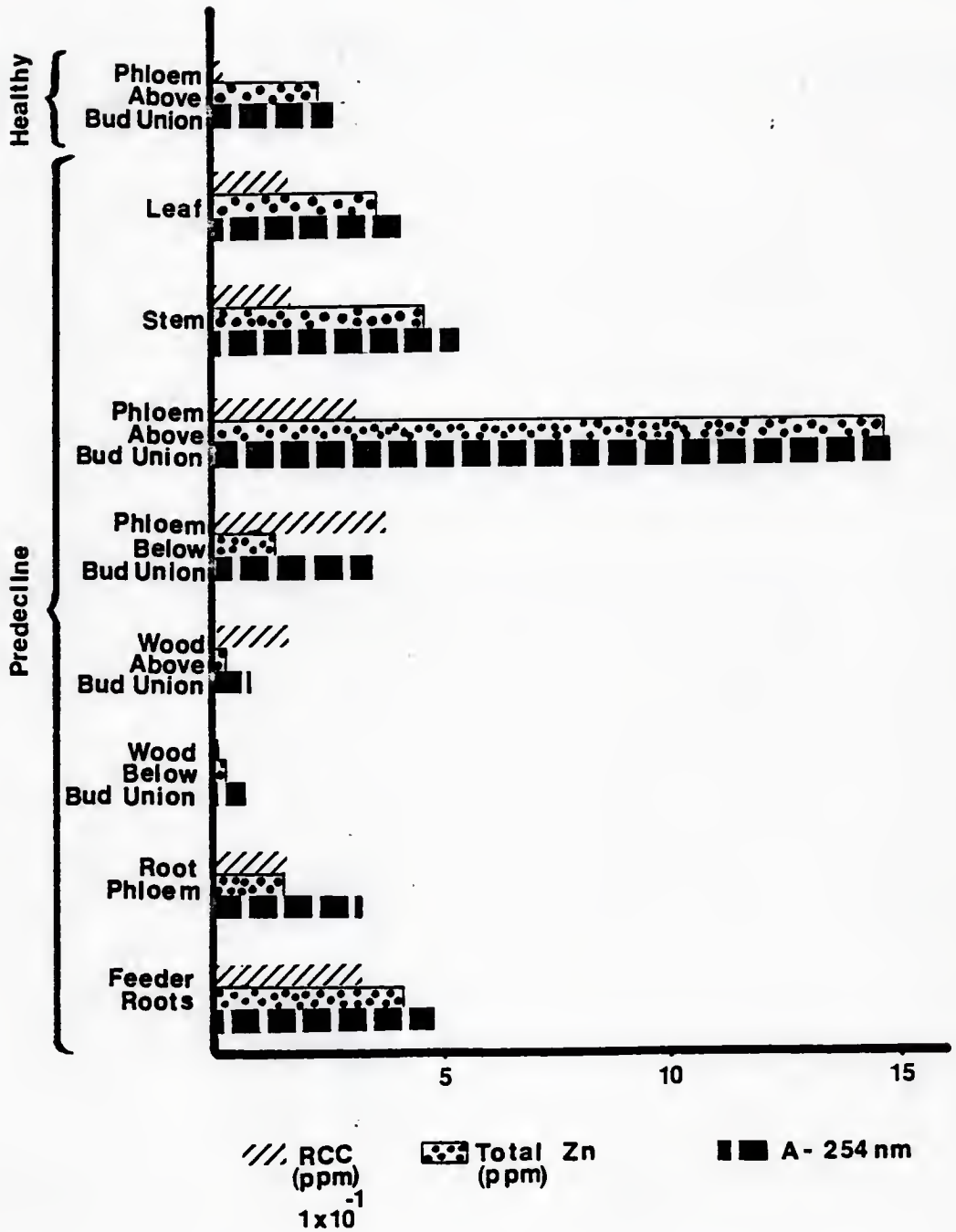
The varied abilities of the 4 species to bind these 3 metals may have had some impact on the low correlation of A-254 nm with TCC. If TCC had been measured on the basis of these 3 (or more) metals, the R values may have been increased.

This experiment would have been better performed on the basis of the relative activities of the three metals in relation to the 4 ligands. This requires more knowledge about the ligand-molecules. Amperometric titration of the ligand with various metals would yield formation constants for each metal, from which activities for each metal could be determined (Hoyle and Thorpe, 1978). To do this experiment, ligand standards are required. The decision as to what should be used as the standards requires knowledge as to the exact nature of the complexing molecules. Therefore the amperometric titrations may have to be performed after amino acid sequencing.

Plant Tissue Localization of Zn-Binding Factor in Predecline Trees

Two replicates were assayed for A-254 nm and 1 for total Zn concentration and RCC to develop preliminary data on the morphological

Figure 17. Comparisons of A-254 nm, ppm total Zn and ppm RCC in healthy phloem tissue and predecline stage, blight-affected leaf, stem, phloem, wood, root phloem and feeder root tissue extract from 'Valencia' sweet orange after IEC on DEAE Sephadex-A-50 with an elution gradient of 0.50-3.0 M NaCl, with subsequent gel filtration on Sephadex G-50 of DEAE-IEC pooled fractions #9-20. These samples were concentrated approximately 5-fold after gel filtration, prior to assay. (All data are given on the basis of extractions made on an equivalent fresh weight basis of 10 g phloem tissue/30 mls 50 mM Tris-HCl buffer, pH 7.8.)


Healthy
Phloem Above Bud Union
Predecline
Leaf
Stem
Phloem Above Bud Union
Phloem Below Bud Union
Wood Above Bud Union
Wood Below Bud Union
Root Phloem
Feeder Roots
5
10
15
RCC (ppm) $^{-1}$ 1x10
Total Zn (ppm)
A - 254 nm

location of the Zn-binding factor accumulating in citrus blight-affected trees at the predecline stage (Figure 17). Eight tissues have been compared graphically to the mean from 2 healthy phloem extracts of tissue removed above the bud union. In general, based on A-254 nm and total Zn, extracts of predecline trunk phloem taken from above the bud union contained at least 3 times the level (on a fresh weight basis) of Zn-binding factor found in all other tissues including healthy trunk phloem from above the bud union. RCC was comparable in all tissues except the wood below the bud union, which had very low RCC. A-254 nm and total Zn assays also indicated that the wood tissues were deficient in Zn-binding factor (as compared to those levels found in healthy tissue). Thus, there is little evidence of the Zn-binding factor in the wood at the predecline stage of blight. This agrees with the lack of Zn in the wood at early predecline (Albrigo et al., 1986).

In addition, the levels of Zn-binding factor (data from all 3 assays) in all tissues excluding phloem above the bud union (> healthy tissue) and wood below the bud union (< healthy tissue) are comparable to that found in healthy tissue. Therefore, Zn-binding factor was accumulating only in the tissue (phloem above the bud union) in which Zn was accumulating at the predecline stage of blight. In relation to this finding, an X-ray microanalytical study of the distribution of Cd in root cross-sections of Zea mays L. has shown Cd accumulation in walls of sieve elements and middle lamella separating the endodermis from the pericycle (Khan et al., 1984). This was in comparison with other tissues in the root cross-section. In the Khan et al. (1984) study, Zn competed well for Cd-sequestering agents. Possibly a normal metal binding factor resides primarily in plant phloem tissues. But with

blight, the predecline tree may manufacture an abnormally high level of the metal-binding molecule.

In addition to the data presented here, 2 samples of soil solution taken proximal to the roots of decline and healthy trees were assayed for RCC. Neither contained significant levels of RCC. This gives some indication that the agent responsible for the overproduction of the Zn-binding factor does not reside in the soil. However, data establishing the absence of elevated A-254 nm associated with Zn are necessary to confirm this. A blight-inducing agent can be transmitted plant to plant (Tucker et al., 1984). It is likely that the transmitted agent is responsible for inducing the synthesis of the Zn-binding factor. Two possible inducing agents are the metal itself, Zn (Grill et al., 1985; 1986; Jackson et al., 1985; Rauser and Glover, 1984), or an organism. Certainly, transmission supports the possibility that the transmitted agent is an organism. However, no such organism has been found consistently at the predecline stage in blight-affected trees. On the other hand, transmission of high Zn levels may be possible, and certainly there is abundant evidence that elevated levels of Zn are present in association with blight. This is not to suggest that one is more likely to be the blight transmitting agent than the other, but is only to suggest that they are both possible transmitting agents.

CHAPTER 4
SUMMARY AND CONCLUSIONS

In this research, 4 anionic species of Zn-binding factors were separated. Two of the anionic species were present in healthy, predecline and decline phloem tissue extracts, while the other two were present only in predecline and decline phloem tissue extracts. This occurred in sweet orange on 2 rootstocks on different sites and grapefruit at a third site. The verity of these findings was shown by the coincidence of A-254 nm, total Zn and RCC data. However, the quantitative values for these assays were not shown to be highly correlated. Assuming that the 4 complexing species are $(\gamma\text{-glu-cys})_n\text{-gly}$, better correlation of the assays may be possible with a γ-polypeptide quantification method (possibly ninhydrin or Ellman's assays). Additionally, the correlation may be improved if the species are treated with saturating levels of Zn and examined separately using the 3 standard assays. Such correlation of the assays is important because A-254 nm and total Zn are the standard assays used in phytochelatin research.

The characterization of the Zn-binding factors revealed several points of circumstantial data which suggest that the isolated species are phytochelatins. First, the 4 complexing species bind Zn, a IIB transition metal which is reportedly sequestered by phytochelatins. In addition the 4 species are capable of complexing other transition metals which are commonly complexed by phytochelatins. Second, they are

temperature stable and pH labile at pH 2 and below. Third, the fractions containing Zn-binding factor absorbed UV at 254 nm but did not at 280 nm. These same fractions had increased levels of total Zn. Fourth, the Zn-binding factor had a low molecular weight of 4kd. Amino acid sequencing is necessary to demonstrate unequivocally that this Zn-binding factor is a phytochelatin.

The pooled fractions containing the Zn-binding factors were shown to be reasonably pure (free of classical proteins) on coomassie-blue stained polyacrylamide gels. Assay of the lanes for A-254 or total Zn as indicators of a Zn-binding factor gave further evidence of the presence of at least 4 anionic species. This agrees with data from the DEAE-IEC (0.25-2.0 M NaCl gradient), where 4 anionic species were separated in predecline samples.

Preliminary data indicated that the Zn-binding factor accumulated in the active phloem above the bud union of a predecline tree up to 3 times the level of the healthy tissue or other tissues in the predecline tree (data was based on equivalent fresh weights of extracted tissues). Wood above and below the bud union of the predecline trees sampled provided no more indication of Zn-binding activity than healthy phloem tissue and in the case of the wood below the bud union there was less. This study should be replicated and performed using corresponding tissue from healthy and decline stage extracts for further verification.

Two Zn-binding factors appear to be normally synthesized metabolites. Other studies suggest that amplified synthesis may be responsible for the production in blight-affected citrus trees of extended forms of the Zn-binding factors which are found in the healthy citrus tree. This is based on the finding that healthy and blight

tissue extracts contained a Zn-binding factor with similar charge in the same fractions (a and b) and on the finding that all the anionic species are very near the same molecular weight. In addition, the new Zn-binding forms in phloem tissue from blight-affected citrus were more highly anionic (c, d and fraction #40) than the existing forms in healthy phloem tissue. The greater anionic character of the c and d forms may be due to addition of cysteine units through the γ-glu-cys unit of the phytochelatin molecule.

Lack of RCC in soil solution taken from the rhizosphere of healthy and decline trees along with transmission data (Tucker et al., 1984) indicates that the agent which induces increased or new production of the Zn-binding factors is probably not of edaphic origin. However, this agent is likely to be Zn or an organism. Either way, the transmitted agent must induce synthesis of an existing or new metabolite in order to form the 4 species separated in this study. It is intriguing that the induced synthesis may be of extended forms of this metal-binding molecule, in view of evidence that the 4 species, obtained from PAGE show varied affinities for Pb, Cd and Zn. This adds further credence to the hypothesis that the 4 species may be several forms of phytochelatin.

With regard to future research, it will be useful to separate the species and collect them in preparative quantity. It is important to use a silver stain which has greater sensitivity, to assay the purity of each isolated complexing agent. Possibly, affinity chromatography will be necessary to achieve optimum purification. With isoelectric focusing, the Zn-binding factors could be further characterized in terms of the isoelectric points of the 4 species. Once optimum purity is

achieved, amino acid composition may be determined. This would provide final verification that the Zn-binding factors associated with citrus blight are phytochelatins. Once the metal-binding molecules have been identified as to content and structure, amperometric titrations may be performed which will enable the comparison of the 4 species as to their relative abilities to bind several metals based on equal activities of those metals.

The effect of the Zn-binding factors on Zn-metabolism and the mechanism by which an altered Zn metabolism affects the manifestation of blight are of interest. This question could be partially addressed by determining if the Zn-binding factors affect the activity of known Zn-requiring enzymes. In addition, determination of the activities of these enzymes in healthy versus predecline and decline trees might contribute evidence as to the directness of the involvement of Zn metabolism in development of the citrus blight syndrome. The integrated consequence of their reduced activity on the tree's metabolism would be much more difficult to ascertain.

An extension of the information about the biosynthesis of the Zn-binding factor is pertinent to blight research. Therefore, determination of whether there is induction of synthesis of new mRNA species or amplification of normal mRNA species is of interest. The data from this study suggest the latter. Further, localization of this increased mRNA within predecline trees should indicate the source-site of the Zn-binding factor.

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BIOGRAPHICAL SKETCH

Kathryn Campbell Taylor was born August 8, 1958, in Greenville, South Carolina. She received an Associate of Arts and Science degree from Southside Virginia Community College in June, 1977. Her Bachelor of Science degree in biology was received from the University of South Carolina in December 1979.

After graduation, Kathryn was employed as a laboratory technician by the South Carolina Department of Agriculture, Columbia, and by Clemson University's Sandhill Experiment Station, Elgin, South Carolina. She managed a greenhouse for initiating vegetable seedlings before transplant to the field, a hydroponic nutrition study and a nutrient analysis laboratory.

She enrolled as a graduate student in the Department of Horticulture at Clemson University in August, 1981. The degree of Master of Science was conferred in December, 1983. Kathryn was accepted for graduate study in the Fruit Crops Department of the University of Florida for January, 1984, enrollment. She will receive the degree of Doctor of Philosophy in May, 1987.

Kathryn was married to William Thomas Taylor, Jr., August, 1979. They have a son, Charles William, born December, 1984, and expect their second child in March, 1987. She plans to continue her research interests in the context of a university environment. She begins a post-doctoral fellowship in the Vegetable Crops Department of the University of Florida, January, 1987.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

L. Gene Albrigo, Chairman
Professor of Horticultural Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

R. Hilton Biggs
Professor of Biochemistry and Molecular Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Christine D. Chase
Assistant Professor of Horticultural Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Karen E. Koch
Associate Professor of Horticultural Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Ivan Stewart
Professor of Biochemistry
and Molecular Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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